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(54) Title: GENETIC DEMONSTRATION OF REQUIREMENT FOR NKX6.1, NKX2.2 AND NKX6.2 IN VENTRAL NEURON GENERATION

(57) Abstract: This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 or Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron. Provided are methods of diagnosing a motor neuron degenerative disease in a subject. Also provides is a method of treating neuronal degeneration in a subjet which comprises implanting in diseased neural tissue of the subject a neural stem cell which is capable of expressing homeodomain Nkx6.1 or Nkx6.2 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

GENETIC DEMONSTRATION OF REQUIREMENT FOR NKX6.1, NKX2.2 AND NKX6.2 IN VENTRAL NEURON GENERATION

This application is a continuation-in-part of U.S. Serial No. 09/654,462, filed September 1, 2000, which is a continuation-in-part of U.S. Serial No. 09/569,259, filed May 11, 2000, the contents of which are hereby incorporated by reference into the present application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

BACKGROUND OF THE INVENTION

During the development of the embryonic central nervous system (CNS) the mechanisms that specify regional identity and neuronal fate are intimately linked (Anderson et al. 1997; Lumsden and Krumlauf 1996; Rubenstein et al. 1998). In the ventral half of the CNS, for example, the secreted factor Sonic hedgehog (Shh) has a fundamental role in controlling both regional pattern and neuronal fate (Tanabe and Jessell 1996; Ericson et al. 19976; Hammerschmidth et al. 1997). Shh appears to function as a gradient signal. In the spinal cord, five distinct classes of neurons can be generated in vitro in response to two- to threefold changes in the concentration of Shh, and the position at which each

neuronal class is generated in vivo is predicted by the concentration required for their induction in vivo (Ericson et al. 1997a; Briscoe et al. 2000). Thus, neurons generated in more ventral regions of the neural tube require progressively higher concentrations of Shh for their induction.

The genetic programs activated in neural progenitor cells in response to Shh signaling, however, remain incompletely defined. Emerging evidence suggests that homeobox genes function as critical intermediaries in the neural response to Shh signals (Lumsden and Krumlauf 1996; Tanabe and Jessell 1996; Ericson et al. 1997; Hammerschmidt et al. 1997; Rubenstein et al. 1998). Several homeobox genes are expressed by ventral progenitor cells, and their expression is regulated by Shh. Gain-of-function studies on homeobox gene action in the chick neural tube have provided evidence that homeodomain proteins are critical for the interpretation of graded Shh signaling and that they function to delineate progenitor domains and control neuronal subtype identity (Briscoe et al. 2000). Consistent with these findings, the pattern of generation of neuronal subtypes in the basal telencephalon and in the ventral-most region of the spinal cord is perturbed in mice carrying mutations in certain Shh-regulated homeobox genes (Ericson et al. 1997; Sussel et al. 1999; Pierani et al., unpublished).

Members of the *Nkx* class of homeobox genes are expressed by progenitor cells along the entire rostro-caudal axis of the ventral neural tube, and their expression is dependent on

Shh signaling (Rubenstein and Beachy 1998). Mutation in the *Nkx2.1* or *Nkx2.2* genes leads to defects in ventral neural pattering (Briscoe et al. 1999; Sussel et al. 1999), raising the possibility that *Nkx* genes play a key role in the control of ventral pattering in the ventral region of the CNS. Genetic studies to assess the role of *Nkx* genes have, however, focused on only the most ventral region of the neural tube. A recently identified *Nkx* gene, *Nkx6.1*, is expressed more widely by most progenitor cells within the ventral neural tube (Pabst et al. 1998; Qiu et al. 1998; Briscoe et al. 1999), suggesting that it may have a prominent role in ventral neural patterning. Here experiments show that in mouse embryos *Nkx6.1* is expressed by ventral progenitors that give rise to motor (MN), V2, and V3 neurons. Mice carrying a null mutation of *Nkx6.1* exhibit a ventral-to-dorsal switch in the identity of progenitor cells and a corresponding switch in the identity of the neuronal subtype that emerges from the ventral neural tube. The generation of MN and V2 neurons is markedly reduced, and there is a ventral expansion in the generation of a more dorsal V1 neuronal subtype. Together, these findings indicate that *Nkx6.1* has a critical role in the specification of MN and V2 neuron subtype identity and, more generally, that *Nkx* genes play a role in the interpretation of graded Shh signaling.

SUMMARY OF THE INVENTION

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention also provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of

the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.

This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention provides a method of converting a stem cell

into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.1 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention provides a method of diagnosing a neurodegenerative disease in a subject which comprises: a) obtaining a suitable sample from the subject; b) extracting nucleic acid from the suitable sample; c) contacting the resulting nucleic acid with a nucleic acid probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker; d) removing unbound labeled nucleic acid probe; and e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1U

Selective changes in homeobox gene expression in ventral progenitor cells in *Nkx6.1* mutant embryos. (Figs. 1A-1C) Expression of *Nkx6.1* in transverse sections of the ventral neural tube of mouse embryos E9.5. (Fig. 1A) Expression of *Nkx6.1* is prominent in ventral progenitor cells and persists in some post-mitotic motor neurons at both caudal hindbrain, E10.5, (Fig. 1B) and spinal cord, E12.5, (Fig. 1C) levels. (Fig. 1D, and 1E) Summary diagrams showing domains of homeobox gene expression in wild-type mouse embryos (Fig. 1D) and the change in pattern of expression of these genes in *Nkx6.1* mutants (Fig. 1E), based on analyses at E10.0 - E12.5. (Figs. 1F-1I) Comparison of the domains of expression of *Nkx6.1* (Figs. 1F, 1J) *Dbx2* (Figs. 1G, 1H, 1K, 1L) and *Gsh1* (Figs. 1I, 1M) in the caudal neural tube of wild-type (Figs. 1F-1I) and *Nkx6.1* mutant (Figs. 1J-1H) embryos. (Fig. 1J) Horizontal lines, approximate position of dorsoventral boundary of the neural tube; vertical lines, expression of *Dbx2* and *Gsh1*. Expression of Sonic hedgehog, *Shh* (Figs. 1N, 1R), *Pax7* (Figs. 1N, 1R), *Nkx2.2* (Figs. 1O, 1S), *Pax6* (Figs. 1P, 1S), *Dbx1* (Figs. 1P, 1T) and *Nkx2.9* (Figs. 1Q, 1U) in wild-type (Figs. 1N-1Q) or *Nkx6.1* mutant (Figs. 1R-1U) embryos at spinal (Figs 1N-1P, 1R-1T) and caudal hindbrain levels (Figs 1Q, 1U). Arrowheads, approximate position of the dorsal limit of *Nkx6.1* expression. Scale bar shown in J= 100μm (Figs. 1A-1C); 50μm (Figs. 1F-1M) or 60μm (Figs. 1N-1U).

Figure 2A-2T

Disruption of motor neuron differentiation in *Nkx6.1* mutant embryos. The relationship between the domain of *Nkx6.1* expression (Figs. 2A-2C, green) by ventral progenitors and the position of generation of motor neurons and V2 interneurons (Figs. 2A-2D) in the ventral spinal cord of E10.5 wild-type embryos. (Fig. 2A) Isl1/2 motor neurons; (Fig. 2B) HB9 motor neurons; (Fig. 2C) *Lhx3* (Lim3) expression (red) by motor neurons, V2 interneurons and their progenitors is confined to the *Nkx6.1* progenitor domain. (Fig. 2D) *Chx10* (green) V2 interneurons coexpress *Lhx3* (red). Expression of *Isl1/2* (Figs. 2E, 2I), *HB9* (Figs. 2F, 2J), *Lhx3* (Figs. 2G, 2K) and *Phox2a/b* (Figs. 2H, 2L) in the ventral spinal cord (Figs. 2E, 2F, 2G) and caudal hindbrain (Fig. 2H) of E10.5 wild-type (Figs. 2E-2H) and *Nkx6.1* mutant (Figs. 2I-2L) embryos. Pattern of expression of *Isl1/2* and *Lhx3* at cervical (Figs. 2M, 2N, 2Q, 2R) and thoracic (Figs. 2O, 2P, 2S, 2T) levels of E12.5 wild-type (Figs. 2M-2P) and Nkx6.1 mutant (Figs. 2Q-2T) embryos. Arrows, position of *Isl1* dorsal D2 interneurons. (Figs. 10Q-10T) Absence, position of *Isl1/2* dorsal D2 interneurons. Scale bar shown in I = 60μm (Figs. 2A-2D); 80μm (Figs. 2E-2L); 120μm (Figs. 2M-2T).

Figures 3A-3J

Motor neuron subtype differentiation in *Nkx6.1* mutant mice. Depletion of both median motor column (MMC) and lateral motor column (LMC) neurons in *Nkx6.1* mutant mice. Expression of *Isl1/2* (red) and *Lxh3* (green) in E12.5 wilt-type (Figs. 3A, 3C) and *Nkx6.1* mutant (Figs. 3B,3D) mice spinal cord at forelimb levels (Figs. 3E-3J). Motor neuron

generation at caudal hindbrain level (Figs. 3E, 3F) *Nkx6.1* expression in progenitor cells and visceral motor neurons in the caudal hindbrain (rhombomere [r] 7/8) of E10.5-E11 wild- type (Fig. 3E) *Nkx6.1* mutant (Fig. 3F) mice. *HB9* expression in hypoglossal motor neurons in E10.5-E11 wild-type mice (Fig. 3G) and *Nkx6.1* mutant (Fig. 3H) mice. Coexpression of *Isl1* (green) and *Phox2a/b* (red) in wild-type (Fig. 3I) or *Nkx6.1* mutant (Fig. 3J) mice. (h) hypoglossal motor neurons; (v) visceral vagal motor neurons. Scale bar shown in C = 50μm (Figs. 3A-3D) or 70μm (Figs. 3E-3J).

Figures 4A-4L

A switch in ventral interneuron fates in *Nkx6.1* mutant mice. *Chx10* expression in V2 neurons at rostral cervical levels of E10.5 wild-type (Fig. 4A) and *Nkx6.1* mutant (Fig. 4B) embryos. En1 expression by V1 neurons at rostral cervical levels of wild-type (Fig. 4C) and *Nkx6.1* mutant (Fig. 4D) embryos. *Pax2* expression in a set of interneurons that includes V1 neurons ((Burrill et al. 1997) at caudal hindbrain levels of wild-type (Fig. 4E) and *Nkx6.1* mutant (Fig. 4F) embryos. (Figs. 4G and 4H) *Sim1* expression by V3 neurons in the cervical spinal cord of wild-type (Fig. 4G) and *Nkx6.1* mutant (Fig. 4H) embryos. *Evx1* expression by V0 neurons at caudal hindbrain levels of wild-type (Fig. 4I) and *Nkx6.1* mutant (Fig. 4J) embryos. *En1* (red) and *Lhx3* (green) expression by separate cell populations in the ventral spinal cord of E11 wild-type (Fig. 4K) and *Nkx6.1* mutant (Fig. 4L) embryos. Scale bar shown in B = 60μm (Figs. 4A-4D); 75μm (Figs. 4E, 4F); 70μm (Figs. 4G, 4J, 4H, 4J), 35μm (Figs. 4K and 4L).

Figure 5A-5B

Changes in progenitor domain identity and neuronal fate in the spinal cord of *Nkx6.1* mutant embryos. (Fig. 5A). In wild-type mouse embryos, cells in the *Nkx6.1* progenitor domain give rise to three classes of ventral neurons: V2 neurons, motor neurons (MN) and V3 neurons. V3 neurons derive from cells in the ventral most region of *Nkx6.1* expression that also express *Nkx2.2* and *Nkx2.9*. V1 neurons derive from progenitor cells that express *Dbx2* but not *Nkx6.1*. (Fig. 5B). In *Nkx6.1* mutant embryos the domain of Dbx2 expression by progenitor cells expands ventrally, and by embyonic day 12 [E12] occupies the entire dorsoventral extent of the ventral neural tube, excluding the floor plate. Checked area indicates the gradual onset of ventral *Dbx2* expression. This ventral shift in *Dbx2* expression is associated with a marked decrease in the generation of V2 neurons and motor neurons and a ventral expansion in the domain of generation of V1 neurons. A virtually complete loss of MN and V2 neurons is observed at cervical levels of the spinal cord. The generation of V3 neurons (and cranial visceral motor neurons at hindbrain levels) is unaffected by the loss of *Nkx6.1* or by the ectopic expression of *Dbx2*.

Figure 6

Human Homeobox Protein Nkx6.1. NCBI Accession No. P78426. (Inoue, H. et al., "Isolation, characterization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Amino acid sequence of human homeobox protein Nkx6.1.

Figure 7

Human NK Homeobox Protein (Nkx6.1) gene, exon 1. NCBI Accession No. U66797. Segment 1 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-682.

Figure 8

Human NK Homeobox Protein (Nkx6.1) gene, exon 2. NCBI Accession No. U66798. Segment 2 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-185.

Figure 9

Human NK Homeobox Protein (Nkx6.1) gene, exon 3 and complete cds. NCBI Accession No. U66799. Segment 3 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-273. Protein encoded is shown in Fig. 7.

Figure 10

Expression of Nkx6.2 and Nkx6.1 in developing mouse and chick spinal cord. (A) At e8.5, Nkx6.2 and Nkx6.1 are

expressed in a broad ventral domain of the mouse neural tube. (B) At e9.0, Nkx6.2 expression is largely confined to a narrow domain immediately dorsal to the domain of Nkx6.1 expression. A few scattered cells that co-express Nkx6.2 and Nkx6.1 are detected in more ventral positions at this stage. (C) At e9.5, Nkx6.2 is expressed in a narrow domain, dorsal to the Nkx6.1 boundary. (D-G) Comparative patterns of expression of Nkx6.2, Nkx6.1, Dbx2, Dbx1 and Pax7 in the intermediate region of e10.5 mouse spinal cord. (H-L) Expression pattern of *Nkx6.2*, *Nkx6.1*, *Dbx2*, *Dbx1* and *Pax7* in HH stage 20 chick spinal cord. Panels on right indicate progenitor domains, defined according to Briscoe et al., 2000.

Figure 11

Elevation in *Nkx6.2* and Dbx2 expression in p1 domain cells in *Nkx6.2* mouse mutants. (A) Diagram of the targeting construct (i) used to replace the coding sequence of *Nkx6.2* (ii) with a tau-lacZ PGK-neo cassette (iii). Red bar indicates region used as probe in genotyping. (B-D) Sagital view of e10.5 spinal cord showing LacZ expression, detected by X-gal staining, in wild type (*wt*) (B) $Nkx6.2^{+/tlz}$ (C) and $Nkx6.2^{tlz/tlz}$ (D) embryos. (E-G) Nkx6.2 and LacZ expression in the p1 domain of *wt* (E), $Nkx6.2^{+/tlz}$ (F), and $Nkx6.2^{tlz/tlz}$ (G) embryos at e10.5. (H-J) In situ hybridization with a 5´-UTR probe shows that expression of *Nkx6.2* is elevated in the p1 domain of $Nkx6.2^{tlz/tlz}$ embryos (J), compared with *wt* (H) or $Nkx6.2^{+/tlz}$ (I) embryos. (K-M) Expression of Dbx2 is up regulated ~2- fold in cells within the p1 domain (yellow bracket) in $Nkx6.2^{tlz/tlz}$ embryos (M), compared with *wt* (K), or $Nkx6.2^{+/tlz}$ (L) embryos. Abbreviations in (A): H=

HindIII, B= BamHI, N= NcoI, S= SphI, A=AccI.

Figure 12

A partial switch from V1 to V0 neuronal fate in *Nkx6.2* mutant mice. (A-E) Expression of Nkx6.2 (A), Nkx6.1 (C, D), Dbx1 (B, C, E), and Pax7 (B) appears normal at caudal hindbrain levels of e10.5 *Nkx6.2*$^{+/tlz}$ embryos. The expression of Nkx6.1 (D) and Dbx1 (E) abuts the ventral and dorsal boundaries of *LacZ* expression. (F-J) In e10.5 *Nkx6.2*$^{tlz/tlz}$ embryos, expression of Nkx6.1 (H, I) and Pax7 (G) is unchanged but expression of Dbx1 (F, G, H) is expanded ventrally into the p1 domain. Many ventral ectopic Dbx1^{+} cells in *Nkx6.2*$^{tlz/tlz}$ embryos express LacZ (J). (K-M) Evx1/2^{+} V0 neurons are generated dorsal to En1^{+} V1 neurons (K) and *LacZ*$^{+}$ cells (M) in *Nkx6.2*$^{+/tlz}$ embryos. En1^{+} neurons express LacZ in *Nkx6.2*$^{+/tlz}$ (L) and *Nkx6.2*$^{tlz/tlz}$ (O) embryos. (N-P) Evx1/2^{+} V0 neurons are generated in increased numbers and at ectopic ventral positions in the caudal hindbrain of *Nkx6.2*$^{tlz/tlz}$ embryos. (N) The number of En1^{+} V1 neurons is reduced and the remaining En1^{+} neurons are intermingled with ectopic Evx1/2^{+} cells. (P) Many Evx1/2^{+} neurons in *Nkx6.2*$^{tlz/tlz}$ embryos co-express LacZ. (Q) Quantitation of Evx1/2^{+} V0, and En1^{+} V1, neurons at the caudal hindbrain of *Nkx6.2*$^{+/tlz}$ and *Nkx6.2*$^{tlz/tlz}$ embryos at e10.5. Counts from 12 sections, mean + S.D. In panels (A-P), the white arrowhead indicates the p0/p1 boundary.

Figure 13

Deregulated expression of Nkx6.2 in *Nkx6.1* mutant mice, and similar patterning activities of Nkx6 proteins in chick neural tube. (A) In e10.5 *wt* embryos, Nkx6.2 expression is

confined to the p1 progenitor domain. (B) In *Nkx6.1*$^{+/-}$ embryos, scattered Nkx6.2^{+} cells are detected in the p2, pMN and p3 domains. (C) In Nkx6.1$^{-/-}$embryos, Nkx6.2 is expressed in most progenitors in the p2, pMN and p3 domains. (D-F) Misexpression of Nkx6.2 at high levels represses the expression of Dbx1 (D) and Dbx2 (E), but not Pax7 (F). (G-P) Expression of Nkx6.2 in dorsal positions of the chick neural tube result in ectopic dorsal generation of motor neurons, as indicated by ectopic induction of Lim3 and HB9 expression (G-I, L-N). Forced expression of Nkx6.2 at high levels in the p0 and p1 progenitor domains promotes the ectopic generation of Chx10^{+} V2 neurons (J, K, O, P) and suppresses Evx1/2^{+} V0 (K, P) and En1^{+} V1 (J, O) neurons.

Figure 14

The deregulated expression of Nkx6.2 underlies motor neuron generation in *Nkx6.1* mutants. (A) In e10.5 *wt* embryos, Nkx6.2 expression is confined to the p1 domain and Nkx6.1 is expressed in the p2, pMN and p3 domains. (B) No change in the expression of Nkx6.1 is detected in *Nkx6.2* $^{tlz/tlz}$ embryos. (C, D) In *Nkx6.1*$^{-/-}$ and *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos, Nkx6.2 expression is derepressed in the p2, pMN and p3 domains. (E) No expression of Nkx6.2 or Nkx6.1 protein is detected in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos. (F, G) HB9^{+}, Isl1/2^{+} motor neurons are generated in normal numbers in *Nkx6.2*$^{tlz/tlz}$ embryos. The number of motor neurons is reduced by ~60% in *Nkx6.1*$^{-/-}$ embryos (H), by ~80% in *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryos (I) and by >90% in *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ at cervical levels of e10.5 spinal cord (J). (K-M) At e12, the number of motor neurons of medial (MMC) (Isl1^{+}, Lim3^{+}) and lateral (LMC) (Isl1^{+}) subtype identity is reduced in

similar proportions in *Nkx6.1*$^{-/-}$ and *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos. Lim3^{+} V2 neurons are missing in *Nkx6.1*$^{-/-}$ embryos and *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos at this stage. (N-P) Quantitation of HB9^{+} and Isl1/2^{+} motor neurons at cervical and lumbar levels in *wt*, *Nkx6.2* and *Nkx6.1* single mutants and in *Nkx6.2*; *Nkx6.1* compound mutants at e10 and e12. Counts from 12 sections, mean + S.D.

Figure 15

Changes in class I protein expression and ventral interneuron generation in *Nkx6* mutants. (A-E) Expression of Nkx6.1 and Nkx6.2 in the spinal cord in different *Nkx6* mutant backgrounds at e10.5. (F-J) Spatial patterns of Pax7 and Dbx2 expression in different *Nkx6* mutant backgrounds. Note that the level of Dbx2 expression in the pMN domain of Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ is very low, implying the existence of a pMN domain restricted gene that has the capacity to repress Dbx2 expression. Recent studies have provided evidence that the bHLH protein Olig2 possesses these properties (Novitch et al., 2001).

(K-O) Spatial patterns of expression of Pax7 and Dbx1 in different *Nkx6* mutant backgrounds. (P-T) Spatial patterns of generation of Evx1/2^{+} V0 neurons and En1^{+} V1 neurons in different *Nkx6* mutant backgrounds. (Q) The generation of V0 neurons expands ventrally into the p1 domain in *Nkx6.2*$^{tlz/tlz}$ mutants at caudal spinal levels. (R, A´) The number of En1^{+} V1 neurons increases ~3-fold in the ventral spinal cord of *Nkx6.1*$^{-/-}$ mutants, and ectopic Evx1/2^{+} cells are detected in position of the pMN domain in these mice (see also Sander et al., 2000). (S, T A´) There is a progressive increase in Evx1/2^{+} V0 neurons and a loss of En1^{+} V1 neurons in the

ventral spinal cord of *Nkx6.1*$^{-/-}$;*Nkx6.2* $^{+/tlz}$ and *Nkx6.1* $^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ embryos. (U,V,Z) The generation of Evx1/2^{+} V0 neurons correlates with the pattern of expression of Dbx1 in progenitors in *wt*, *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ mutant backgrounds. Note that only the most lateral progenitor cells express Dbx1 in Nkx6.1$^{-/-}$; Nkx6.2$^{tlz/tlz}$ embryos, suggesting that expression of Dbx1 in more medially-positioned progenitors is repressed by an as yet undefined gene. (X, Y) Ectopic ventral Evx1^{+} V0 neurons derive from Dbx1^{-} progenitors in *Nkx6.1*$^{-/-}$ and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ mutant embryos. Chx10^{+} V2 neurons are generated at normal numbers in *Nkx6.2*$^{tlz/tlz}$ mutants, but are missing at spinal cord levels in *Nkx6.1*$^{-/-}$, *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ mutants (A´; Figure 5, see Sander et al., 2000).

Figure 16

Dissociation of Dbx expression and V0 neuronal fate in mice with reduced Nkx6 protein activity. (A) In e10.0 *wt* embryos, p0 progenitor cells express Dbx1 and generate Evx1/2^{+} V0 neurons. (B) In e10.0 *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryos there is no change in the domain of expression of Dbx1, but Evx1/2^{+} V0 neurons are generated in lateral positions, along much of the ventral neural tube. (C, D) In Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ embryos examined at e10.0 many ectopic ventral Evx1/2^{+} neurons express LacZ. Framed area in (C) is shown at high magnification in (D) and indicates Evx1/2^{+} neurons that coexpress LacZ. (E) Evx1/2^{+} neurons located at the level of the pMN domain (bracket) derive from progenitors that express low or negligible levels of *Dbx2* mRNA. (F) Summary of Dbx1 expression and V0 neuron

generation in *wt*, *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ embryos. The dissociation of Dbx1 and Evx1/2 expression in *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryo suggests that reduced Nkx6 repressor activity is sufficient to repress Dbx1 but insufficient to repress Evx1 expression.

Figure 17

Genetic interactions between Nkx6 and Dbx proteins during the assignment of motor neuron and interneuron fate in the mouse neural tube. (A) Summary of domains of expression of Nkx6.1 (6.1), Nkx6.2 (6.2), Dbx1 (D1) and Dbx2 (D2) in the ventral neural tube of wild type (wt) and different *Nkx6* mutant embryos. (B) Regulatory interactions between Nkx and Dbx proteins in the ventral neural tube. These interactions result in different levels of Nkx6 protein activity in distinct ventral progenitor domains, and thus promote the generation of distinct neuronal subtypes. For details see text.

Figure 18

Human NK Homeobox Protein (Nkx6.2) gene, complete cds. NCBI Accession No. AF184215.

Figure 19

Human Homeobox Protein Nkx6.2. NCBI Accession No. AAK13251. Amino acid sequence of human homeobox protein Nkx6.2.

Figure 20

Comparison of Amino Acid Sequences of Nkx6.2 Protein of Various Species with Other Nkx Protein Sequences. mNk6.3 =

mouse amino acid sequence of Nkx6.3 protein; rNkx6.1 = rat amino acid sequence of Nkx6.1 protein; mNkx6.2 = mouse amino acid sequence of Nkx6.2 protein; and cNkx6.2 = chick amino acid sequence of Nkx6.2 protein.

5

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the following standard abbreviations are used throughout the specification to indicate specific amino acids:

A=ala=alanine	R=arg=arginine
N=asn=asparagine	D=asp=aspartic acid
C=cys=cysteine	Q=gln=glutamine
E=glu=glutamic acid	G=gly=glycine
H=his=histidine	I=ile=isoleucine
L=leu=leucine	K=lys=lysine
M=met=methionine	F=phe=phenylalanine
P=pro=proline	S=ser=serine
T=thr=threonine	W=trp=tryptophan
Y=tyr=tyrosine	V=val=valine

B=asx=asparagine or aspartic acid

Z=glx=glutamine or glutamic acid

As used herein, the following standard abbreviations are used throughout the specification to indicate specific nucleotides: C=cytosine; A=adenosine; T=thymidine; G=guanosine; and U=uracil.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

In an embodiment of the above-described method of converting a stem cell into a ventral neuron, the nucleic

acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell. In a further embodiment of the method, the nucleic acid is introduced by transfection or transduction. In another further embodiment of the method, the ventral neuron is a motor neuron, a V2 neuron or a V3 neuron.

As used herein, the term "nucleic acid" refers to either DNA or RNA, including complementary DNA (cDNA), genomic DNA and messenger RNA (mRNA). As used herein, "genomic" means both coding and non-coding regions of the isolated nucleic acid molecule. "Nucleic acid sequence" refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both replicating vectors, infectious polymers of DNA or RNA and nonfunctional DNA or RNA.

The nucleic acids of the subject invention also include nucleic acids coding for polypeptide analogs, fragments or derivatives which differ from the naturally-occurring forms in terms of the identity of one or more amino acid residues (deletion analogs containing less than all of the specified residues; substitution analogs wherein one or more residues are replaced by one or more residues; and addition analogs, wherein one or more resides are added to a terminal or medial portion of the polypeptide) which share some or all of the properties of the naturally-occurring forms.

The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA, the complementary DNA strand, and the RNA sequence that is translated into

protein. The nucleic acid includes both the full length nucleic acid sequence as well as non-full length sequences. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell.

As used herein, "protein", "peptide" and "polypeptide" are used to denote two or more amino acids linked by a peptidic bond between the α-carboxyl group of one amino acid and the α-amino group of the next amino acid. Peptide includes not only the full-length protein, but also partial-length fragments. Peptides may be produced by solid-phase synthetic methods that are well-known to those skilled in the art. In addition to the above set of twenty-two amino acids that are used for protein synthesis in vivo, peptides may contain additional amino acids, including but not limited to hydroxyproline, sarcosine, and γ-carboxyglutamate. The peptides may contain modifying groups including but not limited to sulfate and phosphate moieties. Peptides can be comprised of L- or D-amino acids, which are mirror-image forms with differing optical properties. Peptides containing D-amino acids have the advantage of being less susceptible to proteolysis in vivo.

Peptides may by synthesized in monomeric linear form, cyclized form or as oligomers such as branched multiple antigen peptide (MAP) dendrimers (Tam et al. Biopolymers 51:311, 1999). Nonlinear peptides may have increased binding affinity by virtue of their restricted conformations and/or oligomeric nature. Peptides may also

be produced using recombinant methods as either isolated peptides or as a portion of a larger fusion protein that contains additional amino acid sequences.

Peptides may be chemically conjugated to proteins by a variety of well-known methods. Such peptide-protein conjugates can be formulated with a suitable adjuvant and administered parenterally for the purposes of generating polyclonal and monoclonal antibodies to the peptides of interest. Alternatively, unconjugated peptides can be formulated with adjuvant and administered to laboratory animals for the purposes of generating antibodies. Methods for generating and isolating such antibodies are well-known to those skilled in the art.

The nucleic acids of the subject invention include but are not limited to DNA, RNA, mRNA, synthetic DNA, genomic DNA, and cDNA.

The nucleic acid sequence of the Nkx6.2 gene for various species may be found under the following NCBI Accession Nos.: human: AF184215; N55046; N50716N; H49739; H46204; H18874; mouse: BB449783; AV331479; BB358883; BB355466; L08074; and D.melanogaster: AF220236.

The amino acid sequence of the Nkx6.2 protein for various species may be found under the following NCBI Accession Nos.: AAK13251; MXKN2; MXKN1; S35304; T28492; AAF33780; P01524; P01523; 9GSSB; 17GSB; 1BH5D; 4GSSB; 1PGTB; 1GSUB; 1GNWB; 2GLRB; 1AGSB.

As used herein, the term "introducing into a cell" includes but is not limited to transduction and transfection. Transfection can be achieved by calcium phosphate co-precipitates, conventional mechanical procedures such as micro-injection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors or any other method known to one skilled in the art. This invention provides an antibody produced by the above method.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

In an embodiment of the above-described method of diagnosing a motor neuron degenerative disease in a subject the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

As used herein, the term "sample" includes but is not limited to tonsil tissue, lymph nodes, spleen, skin lesions, blood, serum, plasma, cerebrospinal fluid, lymphocytes, urine, transudates, exudates, bone marrow cells, or supernatant from a cell culture.

As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The subjects include but are not limited to mice, rats, dogs, guinea pigs, ferrets, rabbits, chicken and primates. In the preferred embodiment, the subject is a human being.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates

that the subject has the motor neuron degenerative disease.

In an embodiment of the above-described method of diagnosing a motor neuron degenerative disease in a subject the nucleic acid is DNA. In a further embodiment of the above-described method the nucleic acid is RNA. In another embodiment the size fractionation in step (c) is effected by a polyacrylamide or agarose gel. In another embodiment the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label. In yet another embodiment the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

As used herein, "detectable marker" includes but is not limited to a radioactive label, or a calorimetric, a luminescent, or a fluorescent marker. As used herein, "labels" include radioactive isotopes, fluorescent groups and affinity moieties such as biotin that facilitate detection of the labeled peptide. Other labels and methods for attaching labels to compounds are well-known to those skilled in the art.

The phrase "specifically hybridizing" and the phrase "selectively hybridizing" describe a nucleic acid that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectively hybridizing it is meant that a nucleic acid binds to a given target in a manner that is detectable in a different manner from non-target sequence under high

stringency conditions of hybridization. "Complementary", "antisense" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively and specifically hybridize to a nucleic acid. Proper annealing conditions depend, for example, upon a nucleic acid's length, base composition, and the number of mismatches and their position on the nucleic acid, and must often be determined empirically. For discussions of nucleic acid design and annealing conditions for hybridization, see, for example, Sambrook *et al.*(1989) *Molecular Cloning: A Laboratory Manual (2nd ed.)*, Cold Spring Harbor Laboratory, Vols. 1-3 or Ausubel, F., *et al.* (1987) *Current Protocols in Molecular Biology*, New York. The above hybridizing nucleic acids may vary in length. The hybridizing nucleic acid length includes but is not limited to a nucleic acid of at least 15 nucleotides in length, of at least 25 nucleotides in length, or at least 50 nucleotides in length.

This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain

transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

In one embodiment of the above method, the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell. In another embodiment of the above method, the nucleic acid is introduced by transfection or transduction. In a further embodiment of the above method, the ventral neuron is a motor neuron.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.1 in the stem cell so as to thereby convert the stem cell into the ventral neuron. In one embodiment of the above method, the ventral neuron is a motor neuron, a V2 interneuron or a V3 interneuron.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to thereby convert the stem cell into the ventral neuron. In one embodiment of the above method, the ventral neuron is a motor neuron.

This invention provides a method of diagnosing a neurodegenerative disease in a subject which comprises: a) obtaining a suitable sample from the subject; b) extracting nucleic acid from the suitable sample; c) contacting the resulting nucleic acid with a nucleic acid

probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker; d) removing unbound labeled nucleic acid probe; and e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the subject.

In one embodiment of the above method, the suitable sample is spinal fluid. In another embodiment of the above method, the nucleic acid is DNA. In a further embodiment of the above method, the nucleic acid is RNA.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

FIRST SERIES OF EXPERIMENTS

EXPERIMENTAL DETAILS

A. Materials and Methods

Generation of Nkx6.1 null mutation

A null mutation in *Nkx6.1* was generated by using gene targeting in 129-strain ES cells by excising an 800-bp NotI fragment containing part of exon 1 and replacing it by a PGK-neo cassette (Sander and German, unpubl.) Mutants were born at Mendelian frequency and died soon after birth; they exhibited movements only upon tactile stimulation.

Immunocytochemistry and in situ hybridization

Localization of mRNA was performed by in situ hybridization following the method of Schaeren-Wiemers and Gerfin-Moser (1993). The *Dbx2* riboprobe comprised the 5' EcoR1 fragment of the mouse cDNA (Pierani et al. 1999). Probes for other cDNAs were cited in the text and used as described therein. Protein expression was localized by indirect fluorescence immunocytochemistry or peroxidase immunocytochemistry (Briscoe et al. 1999; Ericson et al. 1997). *Nkx6.1* was detected with a rabbit antiserum (Briscoe et al. 1999). Antisera against Shh, Pax7, Isl1/2, HB9, Lhx3, Chx10, Phox2a/b, En1, and Pax2 have been described (Briscoe et al. 1999; Ericson et al. 1997). Fluorescence detection was carried out using an MRC 1024 Confocal Microscope (BioRad).

B. Results and Discussion

To define the role of *Nkx6.1* in neural development, we compared patterns of neurogenesis in the embryonic spinal cord and hindbrain of wild-type mice and mice lacking *Nkx6.1* (Sander et al. 1998). In wild-type embryos, neural expression of *Nkx6.1* is first detected at spinal cord and caudal hindbrain levels at about embryonic day 8.5 (E8.5; Qiu et al. 1998; data not shown), and by E9.5 the gene is expressed throughout the ventral third of the neural tube (Figure 1A). The expression of *Nkx6.1* persists until at least E12.5 (Figures 1B, 1C; data not shown). *Nkx6.1* expression was also detected in mesodermal cells flanking the ventral spinal cord (Figures 1B, 1C). To define more precisely the domain of expression of Nkx6.1, we compared its expressions with that of ten homeobox genes - *Pax3*, *Pax7*, *Gsh1*, *Gsh2*, *Irx3*, *Pax6*, *Dbx1*, *Dbx1*, *Dbx2 and Nkx2.9* - that have been shown to define discrete progenitor cell domains along the dorsoventral axis of the ventral neural tube (Goulding et al. 1991; Valerius et al. 1995; Ericson et al. 1997; Pierani et al. 1999; Briscoe et al. 2000).

This analysis revealed that the dorsal boundary of *Nkx6.1* expression is positioned ventral to the boundaries of four genes expressed by dorsal progenitor cells: *Pax3*, *Pax7*, *Gsh1* and *Gsh2* (Figures 1I, 1N; and data not shown). Within the ventral neural tube, the dorsal boundary of *Nkx6.1* expression is positioned ventral to the domain of *Dbx1* expression and close to the ventral boundary of *Dbx2* expression (Figures 1G, 1H, and 1P). The domain of *Pax6* expression extends ventrally into the domain of *Nkx6.1*

expression (Figure 1O), whereas the expression of *Nkx2.2* and *Nkx2.9* overlaps with the ventral-most domain of *Nkx6.1* expression (Figures 1O, 1Q).

To address the function of *Nkx6.1* in neural development, we analyzed progenitor cell identity and the pattern of neuronal differentiation in *Nkx6.1* null mutant mice (Sander et al. 1998). We detected a striking change in the profile of expression of three homeobox genes, *Dbx2*, *Gsh1* and *Gsh2*, in *Nkx6.1* mutants. The domains of expression of *Dbx2*, *Gsh1* and *Gsh2* each expanded into the ventral neural tube (Figures 1K-1M; data not shown). At E10.5, *Dbx2* was expressed at high levels by progenitor cells adjacent to the floor plate, but at this stage ectopic *Dbx2* expression was detected only at low levels in regions of the neural tube that generate motor neurons (Figure 1K). By E12.5, however, the ectopic ventral expression of *Dbx2* had become more uniform, and now clearly included the region of motor neuron and V2 neuron generation (Figure 1L). Similarly, in *Nkx6.1* mutants, both *Gsh1* and *Gsh2* were ectopically expressed in a ventral domain of the neural tube, and also in adjacent paraxial mesodermal cells (Figure 1M; data not shown).

The ventral limit of Pax6 expression was unaltered in *Nkx6.1* mutants, although the most ventrally located cells within this progenitor domain expressed a higher level of Pax6 protein than those in wild-type embryos (Figures 1O, 1S). We detected no change in the patterns of expression of *Pax3*, *Pax7*, *Dbx1*, *Irx3*, *Nkx2.2*, or *Nkx2.9* in *Nkx6.1* mutant embryos (Figures 1R-1U; data not shown).

Importantly, the level of Shh expression by floor plate cells was unaltered in *Nkx6.1* mutants (Figures 1N and 1R). Thus, the loss of *Nkx6.1* function deregulates the patterns of expression of a selected subset of homeobox genes in ventral progenitor cells, without an obvious effect on Shh levels (Figures 1D, 1E). The role of Shh in excluding *Dbx2* from the most ventral region of the neural tube (Pierani et al. 1999) appears therefore to be mediated through the induction of Nkx6.1 expression. Consistent with this view, ectopic expression of *Nkx6.1* represses *Dbx2* expression in chick neural tube (Briscoe et al. 2000). The detection of sites of ectopic *Gsh1/2* expression in the paraxial mesoderm as well as the ventral neural tube, both sites of *Nkx6.1* expression, suggests that *Nkx6.1* has a general role in restricting *Gsh1/2* expression. The signals that promote ventral *Gsh1/2* expression in *Nkx6.1* mutants remain unclear, but could involve factors other than Shh that are secreted by the notochord (Hebrok et al. 1998).

The domain of expression of *Nkx6.1* within the ventral neural tube of wild-type embryos encompasses the progenitors of three main neuronal classes: V2 interneurons, motor neurons and V3 interneurons (Goulding et al. 1991; Ericson et al. 1997; Qiu et al. 1998; Briscoe et a. 1999, 2000; Pierani et al. 1999; Figures 2A-2D). We examined whether the generation of any of these neuronal classes is impaired in *Nkx6.1* mutants, focusing first on the generation of motor neurons. In *Nkx6.1* mutant embryos there was a marked reduction in the number of spinal motor neurons, as assessed by expression of the homeodomain proteins Lhx3, Isl1/2 and HB9 (Arber et al. 1999; Tsuchida

et al. 1994; Figures 2E-2L), and by expression of the gene encoding the transmitter synthetic enzyme choline acetyltransferase (data not shown). In addition, few if any axons were observed to emerge from the ventral spinal cord (data not shown). The incidence of motor neuron loss, however, varied along the rostrocaudal axis of the spinal cord. Few if any motor neurons were detected at caudal cervical and upper thoracic levels of *Nkx6.1* mutants analyzed at E11-E12.5 (Figures 2M, 2N, 2Q, 2R), whereas motor neuron number was reduced only by 50%-75% at more caudal levels (Figures 2O, 2P, 2S, 2T; data not shown). At all axial levels, the initial reduction in motor neuron number persisted at both E12.5 and p0 (Figures 2M-2T; data not shown), indicating that the loss of *Nkx6.1* activity does not simply delay motor neuron generation. Moreover, we detected no increase in the incidence of TUNEL$^+$ cells in *Nkx6.1* mutants (data not shown), providing evidence that the depletion of motor neurons does not result solely from apoptotic death.

The persistence of some spinal motor neurons in *Nkx6.1* mutants raised the possibility that the generation of particular subclasses of motor neurons is selectively impaired. To address this issue, we monitored the expression of markers of distinct subtypes of motor neurons at both spinal and hindbrain levels of *Nkx6.1* mutant embryos. At spinal levels, the extent of the reduction in the generation of motor neurons that populate the median (MMC) and lateral (LMC) motor columns was similar in *Nkx6.1* mutants, as assessed by the number of motor neurons that coexpressed Isl1/2 and Lhx3 (defining MMC neurons, Figures

3A, 3B) and by the expression of *Raldh2* (defining LMC neurons, Sockanathan and Jessell 1998; Arber et al. 1999; Figures 3C, 3D). In addition, the generation of autonomic visceral motor neurons was reduced to an extent similar to that of somatic motor neurons at thoracic levels of the spinal cord of E12.5 embryos (data not shown). Thus, the loss of *Nkx6.1* activity depletes the major subclasses of spinal motor neurons to a similar extent.

At hindbrain levels, *Nkx6.1* is expressed by the progenitors of both somatic and visceral motor neurons (Figures 3E, 3F; data not shown). We therefore examined whether the loss of *Nkx6.1* might selectively affect subsets of cranial motor neurons. We detected a virtually complete loss in the generation of hypoglossal and abducens somatic motor neurons in *Nkx6.1* mutants, as assessed by the absence of dorsally generated HB9$^+$ motor neurons (Figures 3G, 3H; data not shown, Arber et al. 1999; Briscoe et al. 1999). In contrast, there was no change in the initial generation of any of the cranial visceral motor neuron populations, assessed by coexpression of *Isl1* and *Phox2a* (Briscoe et al. 1999; Pattyn et al. 1997) within ventrally generated motor neurons (Figures 3I, 3J; data not shown). Moroever, at rostral cervical levels, the generation of spinal accessory motor neurons (Ericson et al. 1997) was also preserved in *Nkx6.1* mutants (data not shown). Thus, in the hindbrain the loss of *Nkx6.1* activity selectively eliminates the generation of somatic motor neurons, while leaving visceral motor neurons intact. Cranial visceral motor neurons, unlike spinal visceral motor neurons, derive from progenitors that express the related *Nkx* genes *Nkx2.2* and

Nkx2.9 (Briscoe et al. 1999). The preservation of cranial visceral motor neurons in *Nkx6.1* mutant embryos may therefore reflect the dominant activities of *Nkx2.2* and *Nkx2.9* within these progenitor cells.

We next examined whether the generation of ventral interneurons is affected by the loss of *Nkx6.1* activity. V2 and V3 interneurons are defined, respectively, by expression of *Chx10* and *Sim1* (Arber et al. 1999; Briscoe et al. 1999; Figures 4A, 4G). A severe loss of Chx10 V2 neurons was detected in *Nkx6.1* mutants at spinal cord levels (Figure 4B), although at hindbrain levels of *Nkx6.1* mutants ~50% of V2 neurons persisted (data not shown). In contrast, there was no change in the generation of *Sim1* V3 interneurons at any axial level of *Nkx6.1* mutants (Figure 4H). Thus, the elimination of *Nkx6.1* activity affects the generation of only one of the two major classes of ventral interneurons that derive from the Nkx6.1 progenitor cell domain.

Evx1$^+$, Pax2$^+$ V1 interneurons derive from progenitor cells located dorsal to the *Nkx6.1* progenitor domain, (Figure 4B) within a domain that expresses *Dbx2*, but not *Dbx1* (Burrill et al. 1997; Matise and Joyner 1997; Pierani et al. 1999). Because *Dbx2* expression undergoes a marked ventral expansion in *Nkx6.1* mutants, we examined whether there might be a corresponding expansion in the domain of generation of V1 neurons. In *Nkx6.1* mutants, the region that normally gives rise to V2 neurons and motor neurons now also generated V1 neurons, as assessed by the ventral shift in expression of the En1 and Pax2 homeodomain

proteins (Figures 4B, 4C, 4E, 4F). Consistent with this, there was a two- to threefold increase in the total number of V1 neurons generated in *Nkx6.1* mutants (Figures 4C, 4D). In contrast, the domain of generation of Evx1/2 V0 neurons, which derive from the *Dbx1* progenitor domain (Pierani et al. 1999), was unchanged in *Nkx6.1* mutants (Figures 4I, 4J). Thus, the ventral expansion in *Dbx2* expression is accompanied by a selective switch in interneuronal fates, from V2 neurons to V1 neurons. In addition, we observed that some neurons within the ventral spinal cord of *Nkx6.1* mutants coexpressed the V1 marker *En1* and the V2 marker *Lhx3* (Figures 4K, 4L). The coexpression of these markers is rarely if ever observed in single neurons in wild type embryos (Ericson et al. 1996). Thus, within individual neurons in *Nkx6.1* mutants, the ectopic program of V1 neurogenesis appears to be initiated in parallel with a residual, albeit transient, program of V2 neuron generation. This result complements observations in *Hb9* mutant mice, in which the programs of V2 neuron and motor neuron generation coincide transiently within individual neurons (Arber et al. 1999; Thaler et al. 1999).

Taken together, the findings herein reveal an essential role for the *Nkx6.1* homeobox gene in the specification of regional pattern and neuronal fate in the ventral half of the mammalian CNS. Within the broad ventral domain within which *Nkx6.1* is expressed (Figure 5A), its activity is required to promote motor neuron and V2 interneuron generation and to restrict the generation of V1 interneurons (Figure 5B). It is likely that the loss of motor neurons and V2 neurons is a direct consequence of the

loss of *Nkx6.1* activity, as the depletion of these two neuronal subtypes is evident at stages when only low levels of *Dbx2* are expressed ectopically in most regions of the ventral neural tube. Nonetheless, it can not be excluded that low levels of ectopic ventral Dbx2 expression could contribute to the block in motor neuron generation. Consistent with this view, the ectopic expression of *Nkx6.1* is able to induce both motor neurons and V2 neurons in chick neural tube (Briscoe et al. 2000). V3 interneurons and cranial visceral motor neurons derive from a set of *Nkx6.1* progenitors that also express *Nkx2.2* and *Nkx2.9* (Briscoe et al. 1999, Figure 5A). The generation of these two neuronal subtypes is unaffected by the loss of *Nkx6.1* activity, suggesting that the actions of *Nkx2.2* and *Nkx2.9* dominate over that of Nkx6.1 within these progenitors. The persistence of some spinal motor neurons and V2 neurons in *Nkx6.1* mutants could reflect the existence of a functional homologue within the caudal neural tube.

The role of *Nkx6.1* revealed in these studies, taken together with previous findings, suggests a model in which the spatially restricted expression of *Nkx* genes within the ventral neural tube (Figure 5) has a pivotal role in defining the identity of ventral cell types induced in response to graded Shh signaling. Strikingly, in *Drosophila*, the *Nkx* gene *NK2* has been shown to have an equivalent role in specifying neuronal fates in the ventral nerve cord (Chu et al. 1998; McDonald et al. 1998). Moreover, the ability of *Nkx6.1* to function as a repressor of the dorsally expressed *Gsh1/2* homeobox genes parallels the ability of *Drosophila NK2* to repress *Ind*, a *Gsh1/2*-like

homeobox gene (Weiss et al. 1998). Thus, the evolutionary origin of regional pattern along the dorsoventral axis of the central nervous system may predate the divergence of invertebrate and vertebrate organisms.

5

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SECOND SERIES OF EXPERIMENTS

Introduction

During the development of the vertebrate central nervous system, the assignment of regional identity to neural progenitor cells has a critical role in directing the subtype identity of post-mitotic neurons. Within the ventral half of the neural tube, the specification of progenitor cell identity is initiated by the long-range signalling activity of the secreted factor, Sonic hedgehog (Shh) (Briscoe et al., 2001; Briscoe and Ericson, 2001). Shh signaling appears to establish ventral progenitor cell identities by regulating the spatial pattern of expression of homeodomain transcription factors of the Nkx, Pax, Dbx and Irx families (Ericson et al., 1997; Pierani et al., 1999; Briscoe et al., 2000). Members of all four gene families have been duplicated during evolution (Shoji et al., 1996; Wang et al., 2000; Hoshiyama et al., 1998, Peters et al., 2001), and the resulting homeodomain protein pairs are typically expressed in overlapping or nested domains within the neural tube (Briscoe and Ericson, 2001). Some of these homeodomain protein pairs have been proposed to have distinct, and others redundant, roles in spinal cord patterning (Mansouri and Gruss, 1998; Briscoe et al., 1999; Pierani et al., 2001), but the impact of such homeobox gene duplication on neuronal diversification has not been explored directly.

One unifying feature of this diverse array of progenitor homeodomain proteins is their subdivision into two general

groups, termed class I and II proteins, on the basis of their mode of regulation by Shh signalling (Briscoe and Ericson, 2001). The class I proteins are constitutively expressed by neural progenitor cells, and their expression is repressed by Shh signaling, whereas neural expression of the class II proteins requires exposure to Shh (Ericson et al., 1997; Qiu et al., 1998; Briscoe et al., 1999; 2000; Pabst et al., 2000). Although the spatial pattern of expression of the class I proteins has revealed the existence of five ventral progenitor domains, class II proteins have been identified for only two of these domains (Briscoe et al., 2000), raising questions about the existence and identity of additional class II proteins. There is, however, emerging evidence that the combination of class I and II proteins that is expressed by neural progenitor cells directs the fate of their neuronal progeny. In support of this, misexpression of individual progenitor homeodomain proteins in the chick neural tube promotes the ectopic generation of neuronal subtypes, with a specificity predicted by the normal profile of progenitor homeodomain protein expression (Briscoe et al., 2000; Pierani et al., 2001). Conversely, the analysis of mouse mutants has provided genetic evidence that the activities of specific class I and II proteins are required to establish progenitor cell domains and to direct ventral neuronal fates (Ericson et al., 1997; Briscoe et al., 1999; Sander et al., 2000; Pierani et al., 2001).

The participation of progenitor homeodomain proteins in the conversion of graded Shh signals into all-or-none distinctions in progenitor cell identity depends on cross-

repressive interactions between selected pairs of class I and II protein (Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000; Muhr et al., 2001). In addition, most class I and II proteins have been shown to function directly as transcriptional repressors, through the recruitment of corepressors of the Gro/TLE class (Muhr et al., 2001). These findings have suggested a derepression model of neural patterning which invokes the idea that the patterning activities of individual class I or II proteins are achieved primarily through their ability to repress expression of complementary homeodomain proteins from specific progenitor domains. A central implication of this model is that homeodomain proteins direct progenitor cells to individual neuronal fates by suppressing alternative pathways of differentiation - a view that has strong parallels with proposed mechanisms of lineage restriction during lymphoid differentiation (Nutt et al., 1999; Rolink et al., 1999; Eberhard, et al., 2000).

Much of the evidence that has led to this general outline of ventral neural patterning has emerged from an analysis of members of the *Nkx* gene family. Two closely-related Nkx repressor proteins, Nkx2.2 and Nkx2.9, function as class II proteins that specify the identity of V3 neurons (Ericson et al., 1997; Briscoe et al., 1999, 2000). A more distantly related class II repressor protein, Nkx6.1, is expressed throughout the ventral third of the neural tube and when ectopically expressed, can direct motor neuron and V2 neuron fates (Briscoe et al., 2000; Sander et al., 2000). These gain-of-function studies are supported by an analysis of mice lacking *Nkx6.1* function, which exhibit a virtually

complete failure in V2 interneuron generation (Sander et al., 2000). *Nkx6.1* null mice also show a reduction in motor neuron generation at rostral levels of the spinal cord, but at more caudal levels motor neurons are formed in near-normal numbers (Sander et al., 2000). This observation reveals the existence of an Nkx6.1-independent program of spinal motor neuron generation, although the molecular basis of this alternative pathway is unclear.

A close relative of *Nkx6.1*, termed *Nkx6.2* (also known as *Nkx6B* or *Gtx*), has been identified (Komuro et al., 1993; Lee et al., 2001), and is expressed by neural progenitor cells (Cai et al., 1999). In its alias of Gtx, Nkx6.2 has been suggested to regulate myelin gene expression (Komuro et al., 1993), but its possible functions in neural patterning have not been examined. The identification of an *Nkx6* gene pair prompted us to address three poorly resolved aspects of ventral neural patterning. First, do closely related pairs of repressor homeodomain proteins serve distinct or redundant roles in ventral neural patterning? Second, are class I repressor proteins always complemented by a corresponding class II repressor, and if so, is Nkx6.2 one of the missing class II proteins? Third, to what extent is the generation of spinal motor neurons dependent on the activity of Nkx6 class proteins?

To address these issues we mapped the profile of expression of Nkx6.2 and Nkx6.1 during neural tube development, and analysed mouse *Nkx6* mutants to determine the respective contributions of these two genes to neural patterning. We show that Nkx6.2, like Nkx6.1, functions as a class II

repressor homeodomain protein. Our analysis of *Nkx6* mutants further indicates that the duplication of an ancestral *Nkx6* gene has resulted in the expression of two proteins that exert markedly different levels of repressor activity in the ventral neural tube. This differential repressor activity of these two proteins appears to provide both a fail-safe mechanism during motor neuron generation, and the potential for enhanced diversification of ventral interneuron subtypes. Moreover, we find that under conditions of reduced *Nkx6* gene dosage, ventral neuronal subtypes can be generated from progenitor cells that lack the class I or class II proteins normally required for their generation. This finding supports one of the central tenets of the derepression model of ventral neural patterning - that progenitor homeodomain proteins direct particular neuronal fates by actively suppressing cells from adopting alternative fates.

The specification of neuronal fate in the vertebrate central nervous system appears to depend on the profile of transcription factor expression by neural progenitor cells, but the precise roles of such factors in neurogenesis remain poorly understood. A pair of closely-related homeodomain proteins that function as transcriptional repressors, Nkx6.2 and Nkx6.1, are expressed by progenitor cells in overlapping domains of ventral spinal cord. We provide genetic evidence in the mouse that differences in the level of repressor activity of homeodomain proteins underlies the diversification of ventral interneuron subtypes, and provides a fail-safe mechanism during motor neuron generation. We also show that a reduction in Nkx6 protein

activity permits V0 neurons to be generated from progenitor cells that lack the homeodomain proteins normally required for their generation. This finding provides direct evidence for a model of neuronal fate specification in which progenitor homeodomain proteins direct specific neuronal fates by actively suppressing the expression of transcription factors that direct alternative fates.

EXPERIMENTAL DETAILS

A. Materials and Methods

Generation of *Nkx6.2* mutant mice

Mouse *Nkx6.2* genomic clones were isolated from a 129/Ola mouse genomic library. A targeting construct was constructed by inserting a tau-lacZ/pGKneo cassette into a 5 kb 5' HindIII-NcoI fragment and a 2.7 kb 3' SphI-AccI fragment. The linearized targeting construct was electroporated into E14.1 (129/Ola) ES cells. Cells were selected with G418 and screened by Southern blot analysis using a 200 bp 3' AccI fragment, which detected a 6 kb wild type band and a 2.9 kb mutant band. Recombinant clones were injected into C57BL/6J blastocysts to generate two chimeric founders, both of which transmitted the mutant allele. Mice homozygous for the mutant alleles were born at Mendelian frequency and survived through adulthood. All experiments involved mice maintained on a C57BL/6 background. The generation and genotyping of *Nkx6.1* mutant mice have been described previously (Sander et al. 2000). Compound *Nkx6* mutant mice were obtained by crossing *Nkx6.2*$^{+/tlz}$; *Nkx6.1*$^{+/-}$ double heterozygous mice. Genotyping was performed using Southern blot analysis.

Chick in ovo electroporation

Mouse *Nkx6.2* was isolated by PCR (Komuro et al., 1993) and chick *Nkx6.2* from a chick spinal cord library (Basler et al., 1993) using mouse *Nkx6.1* and *Nkx6.2* as probes. cDNAs encoding full-length mouse and chick *Nkx6.2* were inserted into a RCASBP(B) retroviral vector and electroporated into

the neural tube of stage HH (Hamburger and Hamilton, 1953) 10-12 chick embryos (Briscoe et al., 2000). After 24-48h, embryos were fixed and processed for immunohistochemistry.

Immunohistochemistry and in situ hybridization histochemistry

Immunohistochemical localization of proteins was performed as described (Yamada et al., 1993; Briscoe et al., 2000). Guinea-pig antisera were generated against an 11 amino acid N-terminal sequence of mouse Nkx6.2. Other antibodies used were rabbit anti-Lim3 (Ericson et al., 1997), mAb Hb9 (Tanabe et al., 1998), rabbit anti-Isl1/2 (Tsuchida et al., 1994), rabbit anti-Chx10 (Ericson et al., 1997), rabbit anti-En1 (Davis et al., 1991), mAb anti-Evx1/2, rabbit anti-Dbx1, rabbit anti-Dbx2 (Pierani et al., 1999), rabbit anti-Nkx6.1 (Jörgensen et al., 1999), mAb anti-Pax7 (Ericson et al., 1996), rabbit anti-bgal (Cappel) and goat anti-bgal (Biogeneseis). Images were collected on a Zeiss LSM510 confocal microscope. In situ hybridisation was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using chick probes for *Dbx1*, *Dbx2* (Pierani et al., 1999), *Nkx6.1* (Briscoe et al., 2000) and *Nkx6.2*. A mouse probe for the 5'UTR of *Nkx6.2* comprised 346 bp upstream of the start ATG site. Whole-mount X-gal staining was performed as described (Mombaerts et al., 1996).

B. Results

Distinct patterns of Nkx6.1 and Nkx6.2 expression in embryonic spinal cord

To examine the roles of *Nkx6* class genes in ventral neuronal

specification we compared the patterns of expression of Nkx6.2 and Nkx6.1 with that of other progenitor homeodomain proteins in the spinal cord of mouse and chick embryos. In the caudal neural tube of the mouse, the expression of Nkx6.2 was first detected at ~e8.5, in a broad ventral domain that largely coincided with that of Nkx6.1 (Figure 10A). Between e8.5 and e9.5, the expression of Nkx6.2 was lost from most Nkx6.1$^+$ cells in the ventral neural tube, although expression persisted in a narrow stripe of cells just dorsal to the limit of Nkx6.1 expression (Figure 10B, C). At e10.0-e10.5, virtually all, Nkx6.2$^+$ cells coexpressed Dbx2 (Figure 10E), and the ventral limit of expression of both Nkx6.2 and Dbx2 coincided with the dorsal limit of Nkx6.1 expression at the p1/p2 domain boundary (Figure 10D, E). Nkx6.2 was expressed predominantly within the p1 domain, but scattered Nkx6.2$^+$ cells were detected within the p0 domain - the domain of expression of Pax7$^-$, Dbx1$^+$ cells (Figure 10F). Within the p0 domain, however, individual Nkx6.2$^+$ cells did not coexpress Dbx1, although they did express Dbx2 (Figure 10E-G). Thus, the scattered Nkx6.2$^+$ cells found at the dorsoventral level of the p0 domain exhibit a p1, rather than p0, progenitor cell identity. Studies in chick have similarly shown that p0 and p1 progenitors are interspersed in the most dorsal domain of the ventral neural tube (Pierani et al., 1999).

In the chick neural tube, as in the mouse, *Nkx6.1* and *Nkx6.2* are initially coexpressed in a broad ventral domain (Cai et al., 1999; data not shown). But in contrast to the mouse, *Nkx6.2* expression persists in ventral progenitor cells, with the consequence that the expression of *Nkx6.2* and *Nkx6.1*

also overlaps at later developmental stages (Figure 10H, I). Nevertheless, expression of chick *Nkx6.2* is also detected in a thin stripe of cells dorsal to the limit of *Nkx6.1* expression, within the p1 domain (Figure 10H). Thus, in both species, p1 progenitors coexpress Nkx6.2 and Dbx2 and exclude Nkx6.1.

Nkx6.2 Regulates V0 and V1 Interneuron Fates by Repression of Dbx1 Expression

The establishment and maintenance of progenitor cell domains in the ventral neural tube has been proposed to depend on mutual repressive interactions between complementary pairs of class I and II homeodomain proteins (Briscoe et al., 2000; Muhr et al., 2001). But class II proteins have been identified for only two of the five known progenitor domain boundaries (the p1/p2 and pMN/p3 boundaries) (Ericson et al., 1997; Briscoe et al., 1999, 2000; Sander et al., 2000). The mutually exclusive pattern of expression of Nkx6.2 and Dbx1 within p1 and p0 progenitors led us to consider whether Nkx6.2 might function as a class II protein that represses Dbx1 expression, and thus help to establish the identity of p1 progenitor cells and the fate of their En1$^+$ V1 neuronal progeny.

To test this idea, we analysed the profile of expression of class I and II homeodomain proteins in *Nkx6.2* mutant embryos. We inactivated the mouse *Nkx6.2* gene by homologous recombination in embryonic stem (ES) cells. A targeted *Nkx6.2* allele (*Nkx6.2*^{tlz}) was generated by replacing the coding sequence of *Nkx6.2* with a *tauLacZ* cassette (Figure 11A). In the spinal cord of *Nkx6.2*$^{+/tlz}$ embryos analysed at

e10.5, expression of LacZ and Nkx6.2 coincided within the p1 progenitor domain (see Figure 11E, F). In *Nkx6.2*$^{tlz/tlz}$ embryos, the location of LacZ$^+$ cells was also similar to that in *Nkx6.2*$^{+/tlz}$ embryos (Figure 11F, G), but Nkx6.2 protein was not detected (Figure 11G). These data provide evidence that the *Nkx6.2*^{tlz} allele generates a null mutation, and that disruption of the *Nkx6.2* locus does not perturb the normal spatial pattern of expression of this gene.

We did observe, however, that the level of LacZ expression was markedly elevated in *Nkx6.2*$^{tlz/tlz}$, when compared with *Nkx6.2*$^{+/tlz}$, embryos (Figure 11B-D). An elevation in level of expression of the residual 5' *Nkx6.2* transcript was also detected in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 11H-J). These observations provide evidence that Nkx6.2 negatively regulates its own expression level within p1 progenitor cells.

We next analysed the pattern of expression of class I and II homeodomain proteins in the spinal cord and caudal hindbrain of *Nkx6.2*$^{tlz/tlz}$ embryos. The domains of expression of the class II proteins Nkx2.2 and Nkx6.1, and of the class I proteins Pax7, Dbx2, Irx3 and Pax6 were similar in *Nkx6.2*$^{tlz/tlz}$, *Nkx6.2*$^{+/tlz}$, and wild type embryos (Figure 12B-D, G-I; data not shown). In addition, normal patterns of expression of Dbx2 and Nkx6.1 were detected at the p1/p2 domain boundary (data not shown), showing that establishment of the p1 progenitor domain does not require Nkx6.2 function. However, the level of Dbx2 expression in p1 domain progenitors was increased ~two-fold in Nkx6.2$^{tlz/tlz}$ mutants (Figure 11K-M), indicating that Nkx6.2 normally limits the

level of Dbx2 expression in this domain.

We also detected a marked change in the pattern of expression of the p0 progenitor cell marker Dbx1 in *Nkx6.2*$^{tlz/tlz}$ embryos. At caudal hindbrain levels, the number of ventral Dbx1$^+$ progenitor cells increased 1.7- fold (Figure 12F), and the domain of Dbx1$^+$ cells expanded ventrally, extending through the p1 domain to the dorsal limit of Nkx6.1 expression (Figure 12H). Moreover, in *Nkx6.2*$^{tlz/tlz}$ embryos all of the ectopic Dbx1$^+$ cells found within the p1 domain coexpressed LacZ (Figure 12J). Thus, many progenitors within the p1 domain initiate Dbx1 expression in the absence of Nkx6.2 function. Nevertheless in *Nkx6.2*$^{tlz/tlz}$ embryos, numerous LacZ$^+$ progenitors still lacked Dbx1 expression (Figure 12J), implying the existence of an Nkx6.2-independent means of excluding Dbx1 expression from p1 progenitors. The ventral expansion of Dbx1 was most prominent at caudal hindbrain and cervical spinal levels of the neural tube but a similar, albeit less marked, expansion of Dbx1 expression was detected at caudal spinal levels (data not shown; see Figure 15). Taken together, these data imply that within p1 domain progenitors Nkx6.2 functions as a weak repressor of Dbx2 expression and a more potent repressor of Dbx1 expression.

We next analysed the generation of interneuron subtypes in the ventral neural tube. In wild type embryos, Dbx1$^+$, Dbx2$^+$, Nkx6.2$^-$ p0 progenitors generate Evx1/2$^+$ V0 neurons (Pierani et al., 1999; 2001); Nkx6.2$^+$, Dbx1$^-$, Dbx2$^+$ p1 progenitors give rise to En1$^+$ V1 neurons (Burrill et al., 1997; Ericson et al., 1997), and Nkx6.1$^+$, Irx3$^+$, p2

progenitors give rise to Chx10$^+$ V2 neurons (Ericson et al., 1997; Briscoe et al., 2000). Dbx1 activity in p0 progenitors is required to promote V0 and suppress V1 neuronal fates (Pierani et al., 2001). The ventral expansion in Dbx1 expression in *Nkx6.2*$^{tlz/tlz}$ embryos therefore led us to examine whether the loss of Nkx6.2 function leads progenitor cells within the p1 domain to adopt a V0 rather than V1 neuronal fate.

In the caudal hindbrain of *Nkx6.2* $^{tlz/tlz}$ embryos examined at e10.5, we detected a ~ two-fold increase in the number of Evx1/2$^+$ V0 neurons and the domain of V0 neuronal generation expanded ventrally the normal position of the p1 domain (Figure 12N). Consistent with this, many Evx1/2$^+$ neurons coexpressed LacZ (Figure 12P), showing directly that some V0 neurons derive from p1 progenitors in the absence of Nkx6.2 function. Conversely, the total number of En1$^+$ V1 neurons generated in *Nkx6.2*$^{tlz/tlz}$ embryos was reduced by ~50% (Figure 12Q). The dorsoventral position of generation of the remaining En1$^+$ V1 neurons was similar in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 12N), and these neurons expressed LacZ (Figure 12O) showing directly that Nkx6.2$^+$, Dbx2$^+$ p1 progenitor cells generate V1 neurons. The total number of neurons generated from p1 domain progenitors, defined by Cyn1, TuJ1 and Lim1/2 expression was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos examined at e10.5 (data not shown). In addition, the number of TUNEL$^+$ cells was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos (data not shown). Chx10$^+$ V2 neurons and HB9$^+$, Isl1/2$^+$ motor neurons were present in normal numbers and positions in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 14; data not shown). Together, these findings show that the

activity of Nkx6.2 within p1 progenitors promotes V1 neuronal generation and helps to suppress the generation of V0 neurons, a finding consistent with the proposed role of Nkx6.2 in repressing Dbx1 expression from p1 progenitors.

Repression of Nkx6.2 by Nkx6.1 underlies *Nkx6* gene redundancy in spinal motor neuron generation

We next addressed the respective contributions of *Nkx6.1* and *Nkx6.2* to motor neuron and V2 neuron generation. In the ventral neural tube, p2 and pMN progenitors express Nkx6.1 and give rise to V2 neurons and motor neurons respectively. Ectopic expression of Nkx6.1 is sufficient to induce motor neurons and V2 interneurons in dorsal regions of the neural tube, and in *Nkx6.1* mutant mice V2 neurons are eliminated (Briscoe et al., 2000; Sander et al., 2000). Nevertheless, there is only a partial reduction in motor neuron generation in *Nkx6.1* mutants (Sander et al., 2000), revealing the existence of an *Nkx6.1*-independent pathway of motor neuron generation. Nkx6.2 does not normally contribute to motor neuron specification in the mouse, since its expression is extinguished from ventral progenitors well before the appearance of post-mitotic motor neurons (Figure 10A-C), and there is no change in the number of motor neurons generated in $Nkx6.2^{tlz/tlz}$ embryos (see Figure 14G).

Three lines of evidence, however, led us to consider a cryptic role for Nkx6.2 in motor neuron generation. First, Nkx6.2 and Dbx2 share the same ventral limit of expression at the p1/p2 domain boundary, and the expression of Dbx2 is repressed by Nkx6.1 (Briscoe et al., 2000; Sander et al., 2000). Second, Nkx6.2 negatively regulates its own

expression level within p1 domain progenitors (Figure 11D, G, J). Third, Nkx6.1 and Nkx6.2 possess similar Gro/TLE recruitment activities and DNA target site binding specificities (Muhr et al., 2001). We reasoned therefore that under conditions in which Nkx6.1 activity is reduced or eliminated, Nkx6.2 expression might be derepressed in p2 and pMN progenitors.

In support of this idea, in *Nkx6.1*$^{+/-}$ embryos examined at e10.5 we detected a marked increase in the number of Nkx6.2^{+} cells within the p2 and pMN domains (Figure 13B). And in *Nkx6.1*$^{-/-}$ embryos, expression of Nkx6.2 was detected in virtually all progenitor cells within the p2 and pMN domains (Figure 13C). Indeed, in *Nkx6.1*$^{-/-}$ embryos, the level of Nkx6.2 expression in the nuclei of progenitor cells within the p2 and pMN domains was 1.9-fold greater than that in progenitor cells located within the p1 domain (Figure 13C; data not shown). Together, these data show that Nkx6.1 activity normally represses Nkx6.2 expression from p2 and pMN progenitors in the mouse embryo.

In turn, these findings raised the possibility that in *Nkx6.1*$^{-/-}$ embryos, the derepression of Nkx6.2 expression substitutes for the loss of Nkx6.1 during motor neuron generation. If this is the case, Nkx6.2 would be predicted to mimic the ability of Nkx6.1 to induce motor neurons in vivo. Expression of chick or mouse *Nkx6.2* in the neural tube of HH stage 10-12 chick embryos repressed Dbx2 and Dbx1 expression (Figure 13D-F), and induced ectopic motor neuron differentiation (Figure 13G-I, L-N) with an efficacy similar to that of *Nkx6.1* (Briscoe et al., 2000). These data show that Nkx6.2 can induce ectopic motor neurons when expressed

at high levels in the dorsal neural tube, supporting the idea that both Nkx6 proteins can exert similar patterning activities in vivo (Figure 13D-O; Briscoe et al., 2000). In addition, misexpression of Nkx6.2 in the p0 and p1 progenitor domains suppressed the generation of Evx1/2$^+$ V0 and En1$^+$ V1 neurons and promoted the generation of Chx10$^+$ V2 neurons (Figure 13J, K, O, P). Thus, a high level of expression of Nkx6.2 is not compatible with the generation of either V0 or V1 neurons (Figure 13O, P).

Based on these findings, we examined whether Nkx6.2 has a role in motor neuron generation in *Nkx6.1* mutant mice by testing the impact of removing Nkx6.2 as well as Nkx6.1 on the generation of spinal motor neurons. In *Nkx6.2*$^{tlz/tlz}$ embryos there was no change in the number of motor neurons generated at any level of the spinal cord or hindbrain (Figure 14G,N,O; data not shown). In *Nkx6.1*$^{-/-}$ mutants, the number of spinal motor neurons was reduced by ~60% at cervical levels, but by only 25% at lumbar levels (Figure 14H,N,O, Sander et al., 2000). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos, motor neuron generation was reduced to ~25% of controls at both cervical and lumbar levels (Figure 14I,N,O; data not shown). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos, the generation of motor neurons was reduced to <10% of wild type numbers, at all levels of the spinal cord (Figure 14J). In these *Nkx6* double mutant embryos, residual motor neurons were detected at e10.0, and no further increase in motor neuron number was evident at e12 (Figure 14M, P; data not shown). Since there was no increase in apopototic cell death in the ventral neural tube over this period (data not shown), we infer that the few spinal motor neurons present

in *Nkx6* double mutants are generated prior to e10. Together, these findings demonstrate that Nkx6.2 substitutes for the loss of Nkx6.1 in spinal motor neuron generation, and reveal a link between *Nkx6* gene dosage and the incidence of motor neuron generation.

A Dissociation in Neuronal Fate and Progenitor Cell Identity in *Nkx6* Mutant Mice

We next examined whether a reduction in *Nkx6* gene dosage results in ectopic Dbx protein expression and V1 and V0 neuron generation in the p2 and pMN domains of the ventral spinal cord.

En1$^+$ V1 neurons are normally generated from Dbx2$^+$, Dbx1$^-$ p1 progenitor cells, and we therefore analysed the relationship between Dbx2 expression and En1$^+$ V1 neuronal generation in *Nkx6.1* and *Nkx6.2* compound mutants. As reported previously (Sander et al., 2000), in *Nkx6.1*$^{-/-}$ embryos examined at e10.5, ectopic ventral expression of Dbx2 was detected at high levels in the p2 and p3 domains, although cells in the pMN expressed only very low levels of Dbx2 (Figure 15H; see Sander et al., 2000). Moreover, in *Nkx6.1*$^{-/-}$ embryos, ectopic En1$^+$ neurons were generated in the p2 and pMN domains of the ventral neural tube (Figure 15R). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos, Dbx2 expression was detected at intermediate levels in the pMN domain (Figure 15I), and in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tzl}$ double mutant embryos, Dbx2 was detected at uniformly high levels in the p2 and pMN domains (Figure 15J). Strikingly, in these *Nkx6.1* and *Nkx6.2* compound mutant backgrounds, and despite the enhanced ectopic expression of Dbx2, the number of ectopic ventral

En1$^+$ V1 neurons was reduced rather than increased, when compared with the number generated in *Nkx6.1* single mutants (Figure 15R, T).

Since Evx1$^+$ V0 neurons are normally generated from Dbx1$^+$, Dbx2$^+$ p0 progenitors, we examined whether the reduction in ectopic ventral En1$^+$ V1 neuron generation at low *Nkx6* gene dosage might reflect a change in the pattern of expression of Dbx1, and the ectopic generation of V0 neurons. Consistent with this idea, in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ mutants, scattered Dbx1$^+$ cells were detected in the p2, pMN and p3 domains (Figure 15O), and ectopic ventral Evx1/2$^+$ V0 neurons were detected throughout the ventral neural tube (Figure 15T, Z). Thus, in *Nkx6* double mutants, the loss of V1 neurons is associated with the ectopic ventral expression of Dbx1 and the generation of ectopic V0 neurons.

But in *Nkx6.1* single and *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ compound mutant backgrounds, the normal link between expression of Dbx1 in progenitor cells and the generation of Evx1/2$^+$ V0 neurons was severed. In both these *Nkx6* compound mutants backgrounds, the domain of expression of Dbx1 was unchanged (Figure 15M, N): a result that can be accounted for by the maintained expression of Nkx6.2 within the p1 domain, and the deregulated expression of Nkx6.2 within the p2 and pMN domains. Nevertheless, Evx1/2$^+$ V0 neurons were generated from progenitor cells in the position of p2 and pMN domains, (Figure 15R, S, X, Y).

We next considered whether these ectopic V0 neurons were generated from the position of the p2 and pMN domains, or

whether they simply migrated ventrally from a more dorsal position of origin. Ectopic ventral Evx1/2$^+$ V0 neurons were detected as early as e10.0 (Figure 16B), and many of them coexpressed LacZ (Figure 16C, D), providing evidence that many of these neurons derive from progenitor cells within the position of the p2 and pMN domains. The finding that Evx1/2$^+$ V0 neurons are generated from the pMN domain in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos is especially significant, since these progenitors express negligible levels of Dbx2 (Figure 16E, 17), arguing against the possibility that Dbx2 expression compensates for the absence of Dbx1 during ectopic V0 neuronal generation. These results therefore provide evidence that even though Dbx1 activity is normally required for the generation of V0 neurons (Pierani et al., 2001), under conditions in which *Nkx6* gene dosage is markedly reduced, V0 neurons can be generated from progenitor cells that lack Dbx1 expression.

Nevertheless, the pattern of ventral neurogenesis observed in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ mutants indicated that residual Isl1/2$^+$, HB9$^+$ neurons and ectopic Evx1$^+$ neurons were each generated from progenitors located in the position of the pMN domain. This observation raised the question of whether these two neuronal populations are, in fact, distinct. Strikingly, we found that in this compound *Nkx6* mutant background, many of the residual Isl1/2$^+$, HB9$^+$ neurons transiently expressed Evx1 (Figure 16H, I). Thus, under conditions of reduced *Nkx6* gene dosage, progenitor cells at the position of the pMN domain initially generate neurons with a hybrid motor neuron/V0 neuron identity.

c. Discussion

The patterning of cell types in the ventral neural tube depends on the actions of a set of homeodomain proteins expressed by neural progenitor cells. Duplication of many of these genes has resulted in the overlapping neural expression of pairs of closely-related homeodomain proteins, and raises the question of whether these proteins have distinct or redundant roles during ventral neurogenesis. We have used genetic approaches in mouse to examine the respective contributions of one such homeodomain protein pair, Nkx6.1 and Nkx6.2, in ventral neural patterning. Our results imply that the duplication of an ancestral *Nkx6* gene confers both redundant and distinct roles for Nkx6.1 and Nkx6.2 in ventral neuronal patterning. We discuss below how the specificity and efficacy of Nkx6-mediated transcriptional repression underlies the overlapping divergent patterning activities of the two proteins.

Redundant Activities of Nkx6 Proteins in Motor Neuron and V0 Neuron Generation

Our genetic studies in mice indicate that Nkx6.1 and Nkx6.2 have qualitatively similar activities in promoting the generation of motor neurons and in suppressing the generation of V0 neurons. How are these overlapping patterning activities achieved, given the distinct profiles of expression of these two genes?

Nkx6.1 has been shown to have a role in motor neuron generation (Sander et al., 2000), but the finding that large numbers of motor neurons are generated at caudal levels of

the spinal cord in *Nkx6.1* mutant mice, points to the existence of an Nkx6.1-independent pathway of motor neuron generation. At face value, Nkx6.2 would appear a poor candidate as a mediator of the Nkx6.1-independent pathway of motor neuron specification, since it is not expressed by motor neuron progenitors, nor is motor neuron generation impaired in *Nkx6.2* mutant mice. Nevertheless, the activity of Nkx6.2 is responsible for the efficient generation of spinal motor neurons in *Nkx6.1* mutants. The basis of this redundant function resides in the derepression of Nkx6.2 expression in motor neuron progenitors in *Nkx6.1* mutant mice. Strikingly, Nkx6.2 is even derepressed in *Nkx6.1*$^{+/-}$ embryos, whereas there is no change in the patterns of expression of Dbx2 and other homeodomain proteins implicated in the repression of motor neuron generation. The propensity for Nkx6.2 derepression thus appears to establish a "fail-safe" mechanism that ensures that the net level of Nkx6 protein activity is maintained in motor neuron progenitors under conditions in which Nkx6.1 levels decrease. A similar "fail-safe" regulatory mechanism may operate with other Nkx protein pairs. During pharyngeal pouch development, for example, the loss of Nkx2.6 expression appears to be compensated for by the up-regulation of Nkx2.5 (Tanaka et al., 2000).

The finding that Nkx6.2 is derepressed in the absence of Nkx6.1 function also offers a potential explanation for the divergent patterns of expression of Nkx6.2 in the ventral neural tube of mouse and chick embryos. We infer that the chick *Nkx6.2* gene is not subject to repression by Nkx6.1, permitting its persistent expression in p3, pMN and p2

domain progenitor cells. Thus, in chick, the overlapping functions of Nkx6.1 and Nkx6.2 in motor neuron generation are associated with the coexpression of both genes by motor neuron progenitors, whereas in the mouse, Nkx6.2 activity is held in reserve, through its repression by Nkx6.1.

Nkx6.1 and Nkx6.2 also have an equivalent inhibitory influence on the generation of V0 neurons, albeit through activities exerted in different progenitor domains. In p1 progenitors, the repression of p0 identity and V0 neuron fate is accomplished by Nkx6.2. But ventral to the p1/p2 domain boundary it is Nkx6.1 that prevents Dbx1 expression and V0 neuronal generation. Thus, Nkx6.1 is a potent repressor of Dbx1 expression, despite the fact that these two proteins lack a common progenitor domain boundary. The repression of genes that are normally positioned in spatially distinct domains has been observed with other class I and II proteins (Sander et al., 2000). This feature of neural patterning also parallels the activities of gap proteins in anteroposterior patterning of the *Drosophila* embryo, where the repressive activities of individual gap proteins are frequently exerted on target genes with which they lack a common boundary (Kraut and Levine, 1991; Stanojevic et al., 1991).

Distinct Functions of Nkx6.1 and Nkx6.2 in Ventral Interneuron Generation

We now turn to the question of how Nkx6.1 and Nkx6.2 can exert distinct roles in interneuron generation, given the similarities of the two proteins in DNA target site specificity (Jorgensen et al., 1999; Muhr et al., 2001), and

their overlapping functions in the patterning of motor neurons and V0 neurons.

One factor that contributes to the opponent influence of Nkx6.1 and Nkx6.2 on the specification of V1 interneuron fate is a distinction in the dorsal limit of expression of the two proteins in the neural tube, presumably a reflection of differences in the regulation of expression the two proteins by graded Shh signalling. Nkx6.1 expression stops at the p1/p2 domain boundary. And within the p2 domain, Nkx6.1 suppresses p1 progenitor identity through repression of Dbx2 and Nkx6.2 expression, in this way ensuring the generation of Chx10$^+$ V2 neurons. Nkx6.2, in contrast, occupies the p1 domain, where it is coexpressed with Dbx2. In p1 domain cells, Nkx6.2 promotes the generation of En1$^+$ V1 neurons by repressing the expression of Dbx1 and Evx1, determinants of V0 neuronal fate (Pierani et al., 2001; Moran-Rivard et al., 2001). Nevertheless, only a fraction of p1 progenitors initiate Dbx1 expression and acquire V0 neuron fate in the absence of Nkx6.2 function, raising the possibility that Dbx2 may also have a role in repressing Dbx1 expression within p1 progenitors (see Pierani et al., 1999).

The second major factor that underlies the opponent activities of Nkx6.1 and Nkx6.2 in V1 interneuron specification appears to be a difference in the potency with which the two Nkx6 proteins repress a common set of target genes. This view is supported by several observations. Nkx6.1 completely represses Nkx6.2, whereas Nkx6.2 exerts an incomplete negative regulation of its own expression in

p1 domain progenitors. Thus, Nkx6.1 is evidently a better repressor of Nkx6.2 than is Nkx6.2 itself. Similarly, Nkx6.2 is coexpressed with Dbx2 in p1 domain progenitors, whereas Nkx6.1 excludes Dbx2 from p2 domain progenitors, indicating that Nkx6.1 also is a more effective repressor of Dbx2 expression than is Nkx6.2. Consistent with this view, Nkx6.2 fails to repress Dbx2 expression completely from ventral progenitors in *Nkx6.1* mutants. The fact that Nkx6.2 is only a weak repressor of Dbx2 is critical for the formation of the p1 domain, since the maintained expression of Dbx2 in these cells ensures the exclusion of Nkx6.1 expression (Briscoe et al., 2000).

Our results do not resolve why Nkx6.2 is a weaker repressor than Nkx6.1 in vivo. Differences in the primary structure of Nkx6.2 and Nkx6.1 (Cai et al., 1999; Muhr et al., 2001) could result in an intrinsically lower repressor activity of Nkx6.2, when compared with that of Nkx6.1. But our findings are also consistent with the possibility that the two Nkx6 proteins have inherently similar repressor activities, and that the Nkx6.2 protein is merely expressed at a lower level. Indeed within p1 progenitors, the level of Nkx6.2 expression is clearly subject to tight regulation, with significant consequences for neuronal specification. The selective expression of Nkx6.2 in p1 progenitors, coupled with its weak negative autoregulatory activity, ensures a level of Nkx6 activity that is low enough to permit Dbx2 expression but is still sufficient to repress Dbx1 expression, thus promoting the generation of V1 neurons.

Our findings therefore reveal that a gradient of extracellular Shh signalling is translated intracellularly into stepwise differences in the level of Nkx6 activity along the ventral-to-dorsal axis of the neural tube. Moreover, the different Nkx6 protein activity levels within ventral progenitor cells are a critical determinant of ventral neuronal fate. Cells that express low or negligible levels of Nkx6 activity (p0 progenitors) are directed to a V0 neuronal fate, cells that express an intermediate Nkx6 activity level (p1 progenitors) are directed to a V1 fate, and cells that express a high Nkx6 activity level (pMN and p2 progenitors) are directed to a motor neuron or V2 fate (Figure 17).

Nkx6 Repressor Function and Neuronal Patterning by Derepression

The finding that many progenitor homeodomain proteins exert mutual-cross repressive interactions has led to a model of spinal neuronal patterning based on transcriptional derepression (Muhr et al., 2001). Similar cross-repressive interactions may establish regional progenitor domains in more rostral regions of the developing CNS (Toresson et al., 2000; Yun et al., 2001). A premise of this model is that transcriptional repression is exerted at two sequential steps in neurogenesis. One repressive step operates at the level of the progenitor homeodomain protein themselves, but a second repressive step is exerted on neuronal subtype determinant factors that have a downstream role in directing neuronal subtype fates (Briscoe et al., 2000; Muhr et al., 2001).

Our analysis of *Nkx6* compound mutant mice provides direct support for this two-step repression model, and in addition indicates that progenitor homeodomain proteins and neuronal subtype determinants differ in their sensitivity to repression by the same class II protein. Normally, the functions of *Dbx1* and *Evx1* are required sequentially during the generation of V0 neurons (Pierani et al., 2001; Moran-Rivard et al., 2001). In *Nkx6.1*$^{-/-}$; *Nkx6.2* $^{+/tlz}$ mutants, however, the generation of Evx1/2^{+} V0 neurons occurs in the absence of expression of Dbx1 by neural progenitor cells. Dbx1 expression is therefore dispensable for V0 neuron generation under conditions of reduced *Nkx6* gene dosage. From these results, we infer that the net level of Nkx6 protein activity in ventral progenitor cells is still above threshold for repression of Dbx1 expression, but is below the level required for repression of Evx1 expression. These data therefore support the idea that Nkx6 proteins normally inhibit V0 neuronal fate by repressing the class I progenitor homeodomain protein Dbx1, and independently by repressing expression of the V0 neuronal subtype determinant Evx1.

A differential sensitivity of progenitor homeodomain proteins and neural subtype determinants to repression appears therefore to underlie the dissociation of progenitor cell identity and neuronal fate observed in *Nkx6* mutants. Such two-tiered repression is, in principle, necessary to specify neuronal fate through transcriptional derepression. In the case of Nkx6.1, for example, repression of Dbx1 and Dbx2 (and possible other unidentified repressors) should be sufficient to derepress motor neuron subtype determinants such as MNR2 and Lim3 in pMN progenitors. But, unless Nkx6.1

also represses the expression of V0 determinants, Evx1 expression would also be initiated in differentiating motor neurons, resulting in a hybrid neuronal phenotype. Indeed, under conditions in which *Nkx6* gene dosage is reduced or eliminated, some of the neurons generated from the position of the pMN domain do transiently express a hybrid motor neuron/V0 neuron phenotype.

The derepression model also invokes the idea that a major role of Nkx6 class proteins is to exclude the expression of Dbx2 and other proteins that inhibit motor neuron generation. This view offers a potential explanation of why a few residual motor neurons are generated in *Nkx6* double mutants. We find that in the absence of *Nkx6* gene function, residual motor neurons are generated only at early developmental stages, suggesting that progenitor cells within the position of the pMN domain have committed to a motor neuron fate prior to the onset of the deregulated ventral expression of Dbx2 and other motor neuron repressors. We note that a third *Nkx6*-like gene exists in the mouse, but this gene is not expressed in the spinal cord of wild type or *Nkx6* mutant embryos (E. Anderson and J. Ericson, unpublished data), and thus its activity appears not to account for the residual motor neurons generated in *Nkx6* double mutants. Importantly, the detection of residual motor neurons in *Nkx6* double mutants also provides evidence that Nkx6 proteins do not have essential functions as transcriptional activators during motor neuron specification, further supporting their critical role as repressors.

Finally, the present studies and earlier work on neurogenesis in the ventral spinal cord (Ericson et al., 1996; Thaler et al., 1999; Arber et al., 1999; Sander et al., 2000) have provided evidence that newly-generated neurons can sometimes express mixed molecular identities. These observations raise the possibility that repressive interactions that select or consolidate individual neuronal identities are not restricted to progenitor cells. Consistent with this view, Evx1 is required to establish V0 and repress V1 neuronal identity through an action in post-mitotic neurons (Moran-Rivard et al., 2001), although it remains unclear whether Evx1 itself functions in this context as an activator or repressor. Similarly, the homeodomain protein HB9 has been implicated in the consolidation of motor neuron identity, through repression of V2 neuronal subtype genes (Arber et al., 1999; Thaler et al., 1999). HB9 possesses an eh-1 Gro/TLE recruitment domain (Muhr et al., 2001), suggesting that HB9 controls the identity of post-mitotic motor neurons through a direct action as a transcriptional repressor. The consolidation of neuronal subtype identity in the spinal cord may therefore depend on transcriptional repressive interactions within both progenitor cells and post-mitotic neurons.

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Yamada, T., Pfaff, S.L., Edlund, T., and Jessell, T.M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell 73, 673-686.

Yun, K., Potter, S. and Rubenstein, J.L. (201. Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development *128*, 193-205.

What is claimed is:

1. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

2. The method of claim 1, wherein the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell.

3. The method of claim 1, wherein the nucleic acid is introduced by transfection or transduction.

4. The method of claim 1, wherein the ventral neuron is a motor neuron, a V2 interneuron or a V3 interneuron.

5. A method of diagnosing a motor neuron degenerative disease in a subject which comprises:

a) obtaining a nucleic acid sample from the subject;

b) sequencing the nucleic acid sample; and

c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid

sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

6. The method of claim 5, wherein the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

7. A method of diagnosing a motor neuron degenerative disease in a subject which comprises:

a) obtaining a nucleic acid sample from the subject;

b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes;

c) separating the resulting nucleic acid fragments by size fractionation;

d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands;

d) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and

f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.

8. The method of claim 7, wherein the nucleic acid is DNA.

9. The method of claim 7, wherein the nucleic acid is RNA.

10. The method of claim 7, wherein the size fractionation in step (c) is effected by a polyacrylamide or agarose gel.

11. The method of claim 7, wherein the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label.

12. The method of claim 7, wherein the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

13. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

14. The method of claim 13, wherein the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell.

15. The method of claim 13, wherein the nucleic acid is introduced by transfection or transduction.

16. The method of claim 13, wherein the ventral neuron is a motor neuron.

17. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.1 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

18. The method of claim 17, wherein the ventral neuron is a motor neuron, a V2 interneuron or a V3 interneuron.

19. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

20. The method of claim 19, wherein the ventral neuron is a motor neuron.

21. A method of diagnosing a neurodegenerative disease in a subject which comprises:

a) obtaining a suitable sample from the subject;

b) extracting nucleic acid from the suitable sample;

c) contacting the resulting nucleic acid with a nucleic acid probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker;

d) removing unbound labeled nucleic acid probe; and

e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the subject.

22. The method of claim 21, wherein the suitable sample is spinal fluid.

23. The method of claim 21, wherein the nucleic acid is DNA.

24. The method of claim 21, wherein the nucleic acid is RNA.

PCT/US01/27256

Figure 1

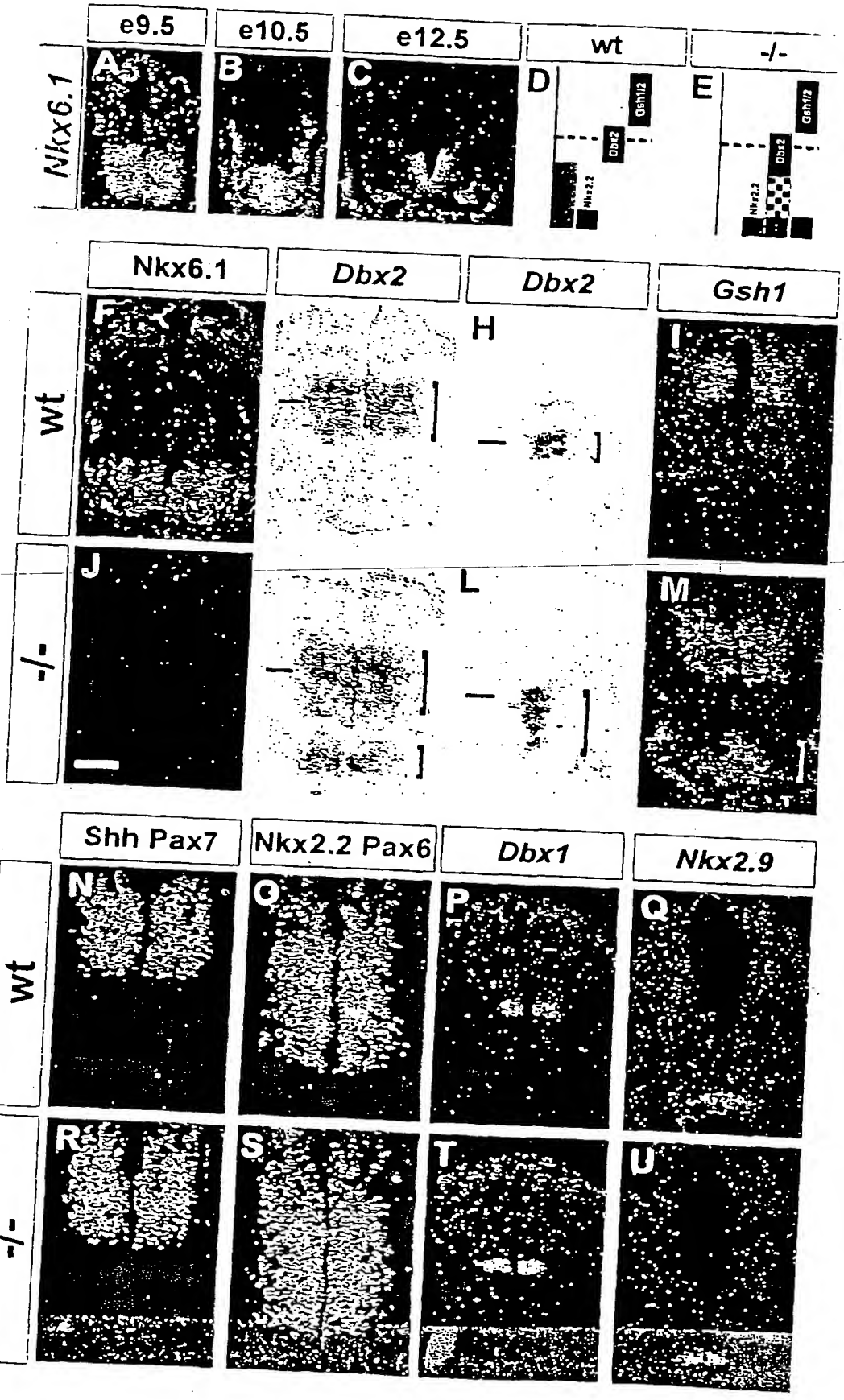

Figure 2

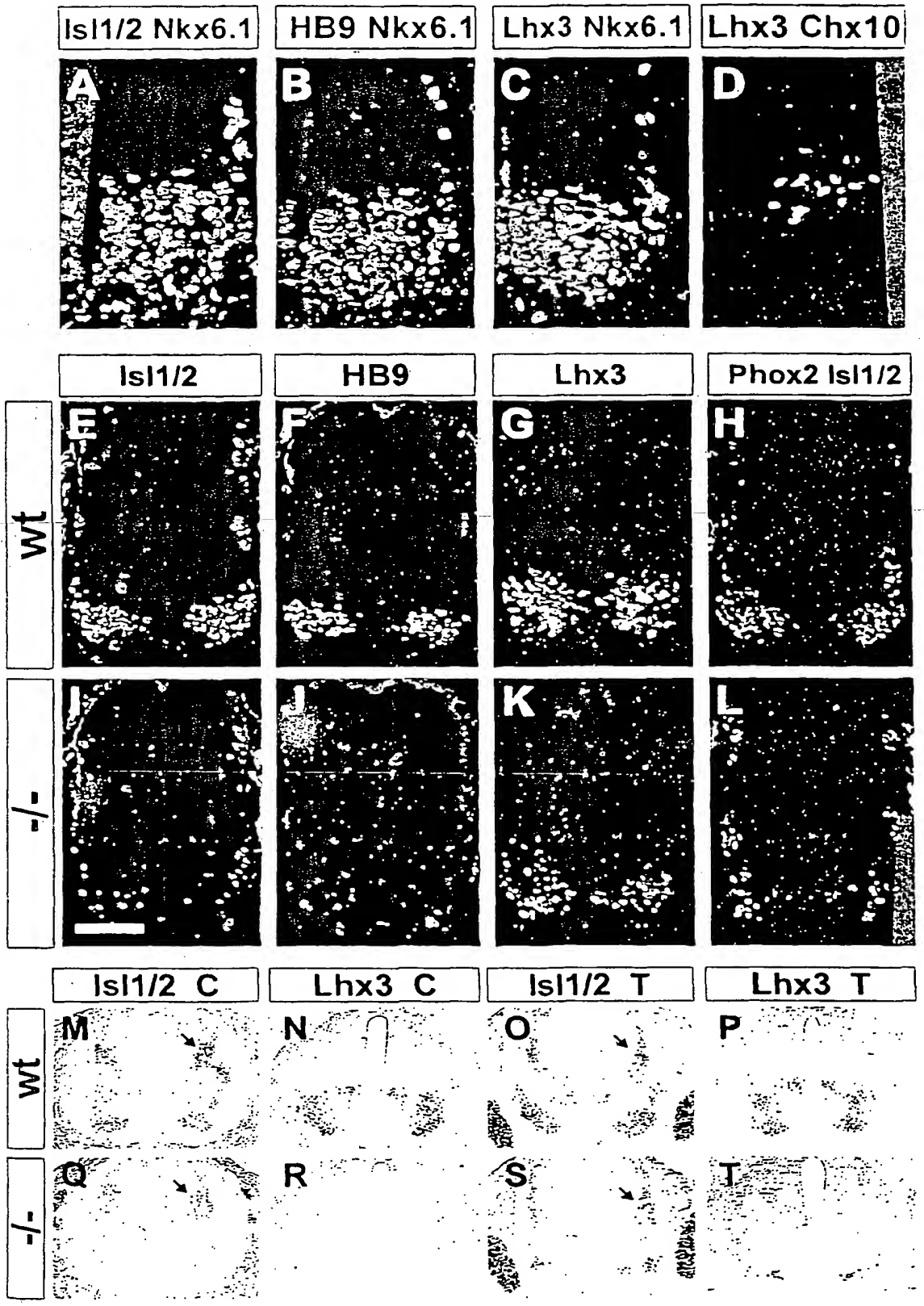
Isl1/2 Nkx6.1
HB9 Nkx6.1
Lhx3 Nkx6.1
Lhx3 Chx10
A
B
C
D
Isl1/2
HB9
Lhx3
Phox2 Isl1/2
wt
-/-
E
F
G
H
I
J
K
L
Isl1/2 C
Lhx3 C
Isl1/2 T
Lhx3 T
wt
-/-
M
N
O
P
Q
R
S
T

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Figure 3

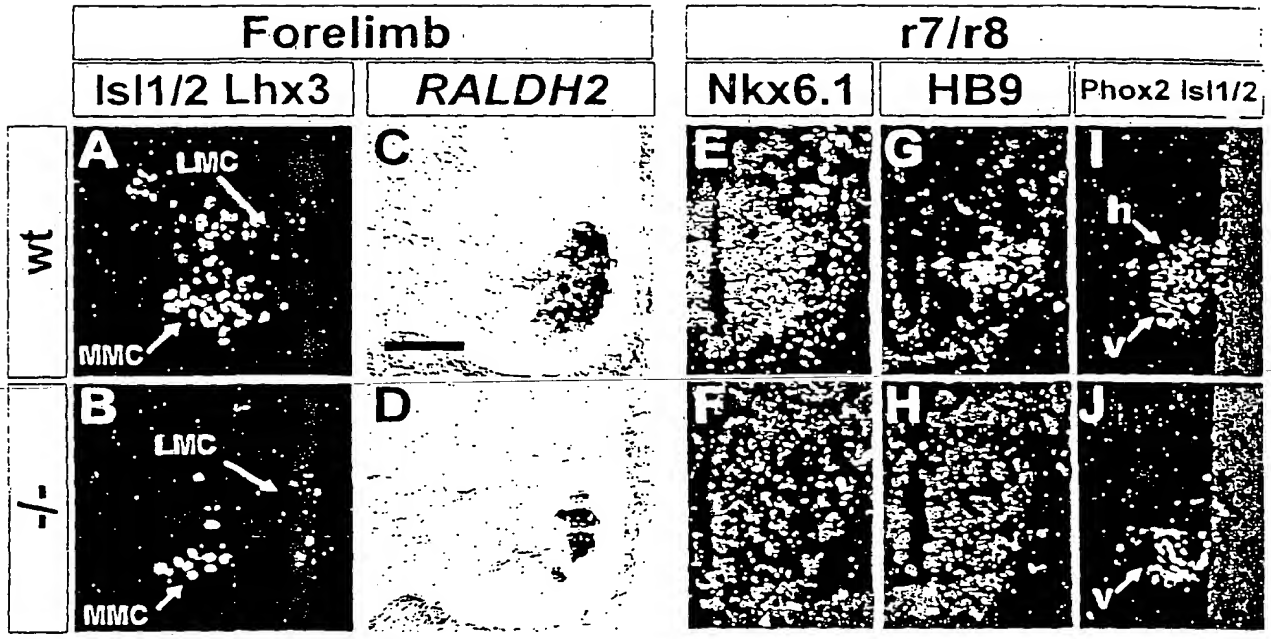
Forelimb
r7/r8
Isl1/2 Lhx3
RALDH2
Nkx6.1
HB9
Phox2 Isl1/2
wt
-/-
A
LMC
MMC
B
LMC
MMC
C
D
E
F
G
H
I
h
v
J
v

Figure 4

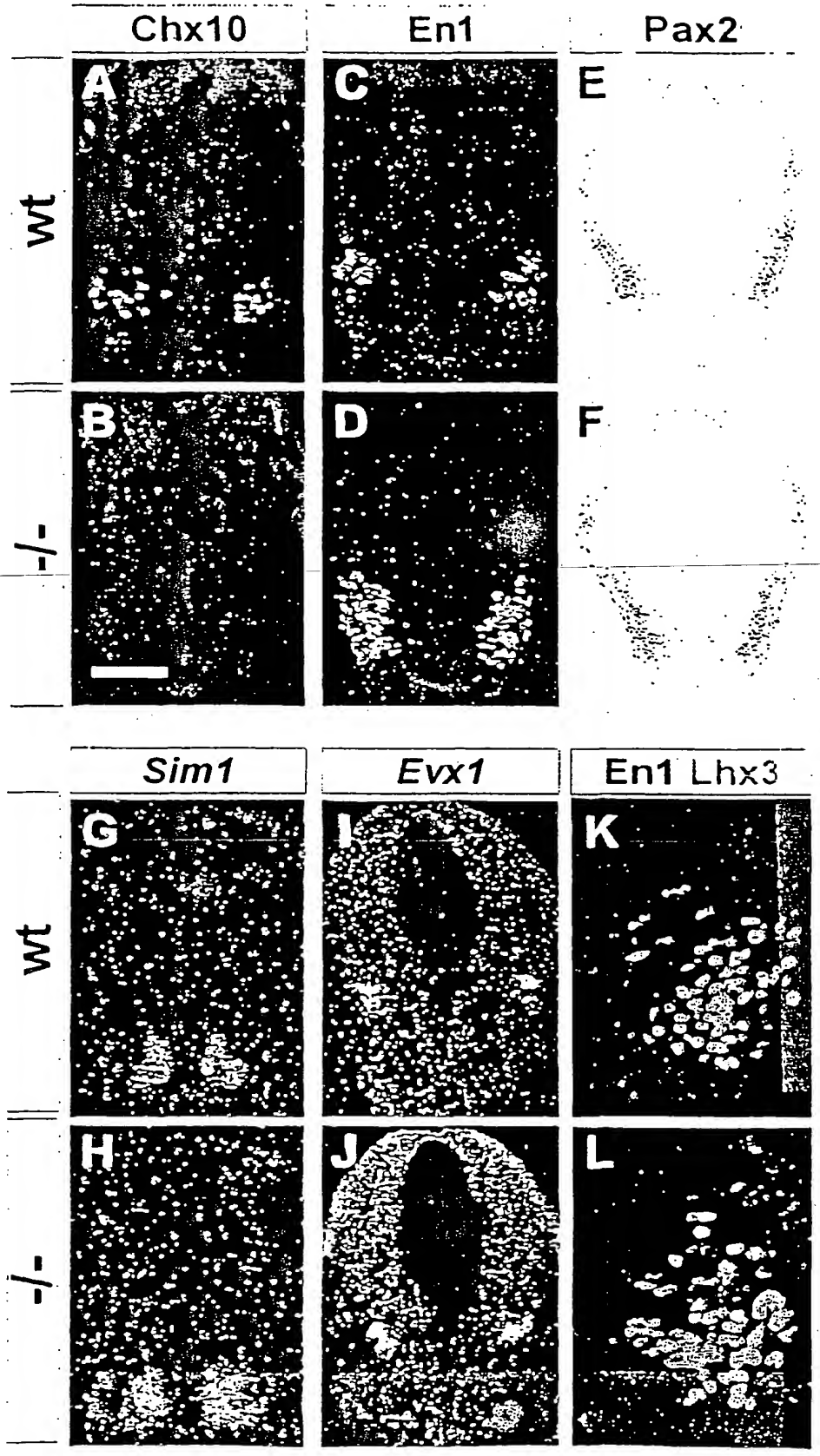

Figure 5

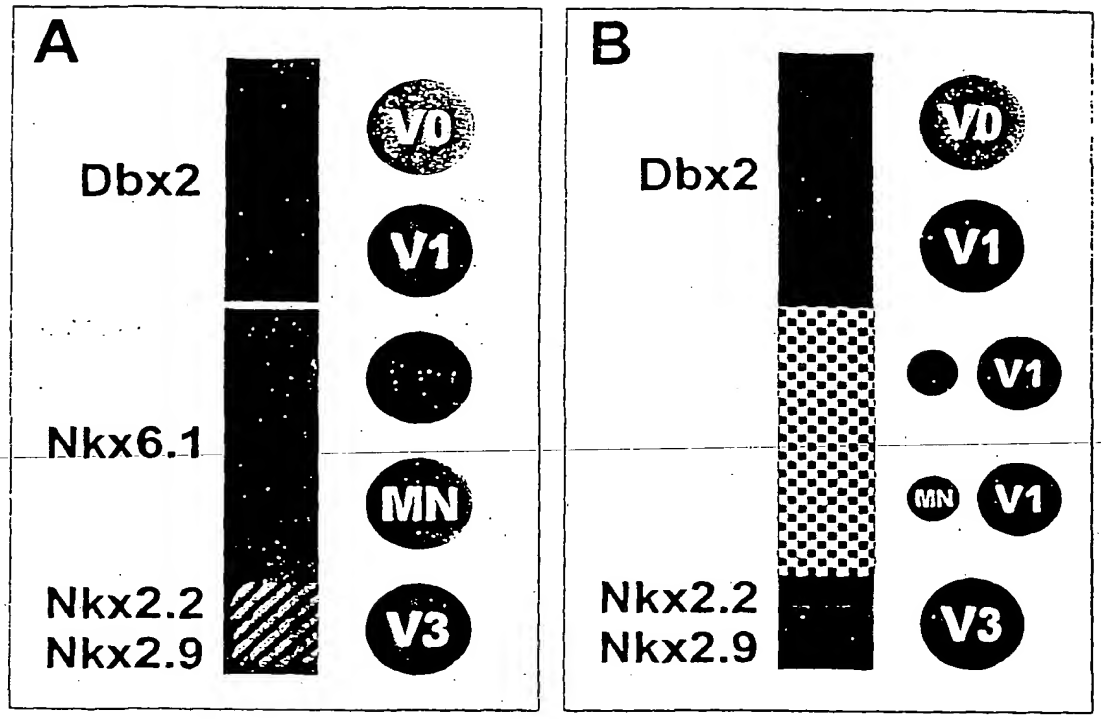
A
Dbx2
Nkx6.1
Nkx2.2
Nkx2.9
V0
V1
MN
V3
B
Dbx2
Nkx2.2
Nkx2.9
V0
V1
V1
MN
V1
V3

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Figure 6

```
  1 mlavgamegt rqsafllssp plaalhsmae mktplypaay pplpagppss sssssssssp
 61 spplgthnpg glkppatggl sslgsppqql saatphginn ilsrpsmpva sgaalpsasp
121 sgssssssss asassasaaa aaaaaaaaaa sspagllagl prfsslsppp pppglyfsps
181 aaavaavgry pkplaelpgr tpifwpgvmq sppwrdarla ctphqgsill dkdgkrkhtr
241 ptfsgqqifa lektfeqtky lagperarla yslgmtesqv kvwfqnrrtk wrkkhaaema
301 takkkqdset erlkgasene eedddynkpl dpnsddekit qllkkhksss ggggglllha
361 sepesss
```

Figure 7

```
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  61 agccctcccc tggccgccct gcacagcatg gccgagatga agaccccgct gtaccctgcc
 121 gcgtatcccc cgctgcctgc cggccccccc tcctcctcgt cctcgtcgtc gtcctcctcg
 181 tcgccctccc cgcctctggg cacccacaac ccaggcggcc tgaagccccc ggccacgggg
 241 gggctctcat ccctcggcag cccccccgcag cagctctcgg ccgccacccc acacggcatc
 301 aacaatatcc tgagccggcc ctccatgccc gtggcctcgg gggccgccct gccctccgcc
 361 tcgccctccg gttcctcctc ctcctcttcc tcgtccgcct ctgcctcctc cgcctctgcc
 421 gccgccgcgg ctgctgccgc ggccgcagcc gccgcctcat ccccggcggg gctgctggcc
 481 ggactgccac gctttagcag cctgagcccg ccgccgccgc cgcccgggct ctacttcagc
 541 cccagcgccg cggccgtggc cgccgtgggc cggtacccca agccgctggc tgagctgcct
 601 ggccggacgc ccatcttctg gcccggagtg atgcagagcc cgccctggag ggacgcacgc
 661 ctggcctgta cccctcgtga gt
```

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Figure 8

```
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 61 ctttttccgg acagcagatc ttcgccctgg agaagacttt cgaacaaaca aaatacttgg
121 cggggcccga gagggctcgt ttggcctatt cgttggggat gacagagagt caggtcaagg
181 tgagt
```

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Figure 9

```
  1 cctcaggtct ggttccagaa ccgccggacc aagtggagga agaagcacgc tgccgagatg
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121 gaggaagagg acgacgacta caataagcct ctggatccca actcggacga cgagaaaatc
181 acgcagctgt tgaagaagca caagtccagc agcggcggcg gcggcggcct cctactgcac
241 gcgtccgagc cggagagctc atcctgaacg ccg
```

FIGURE 10

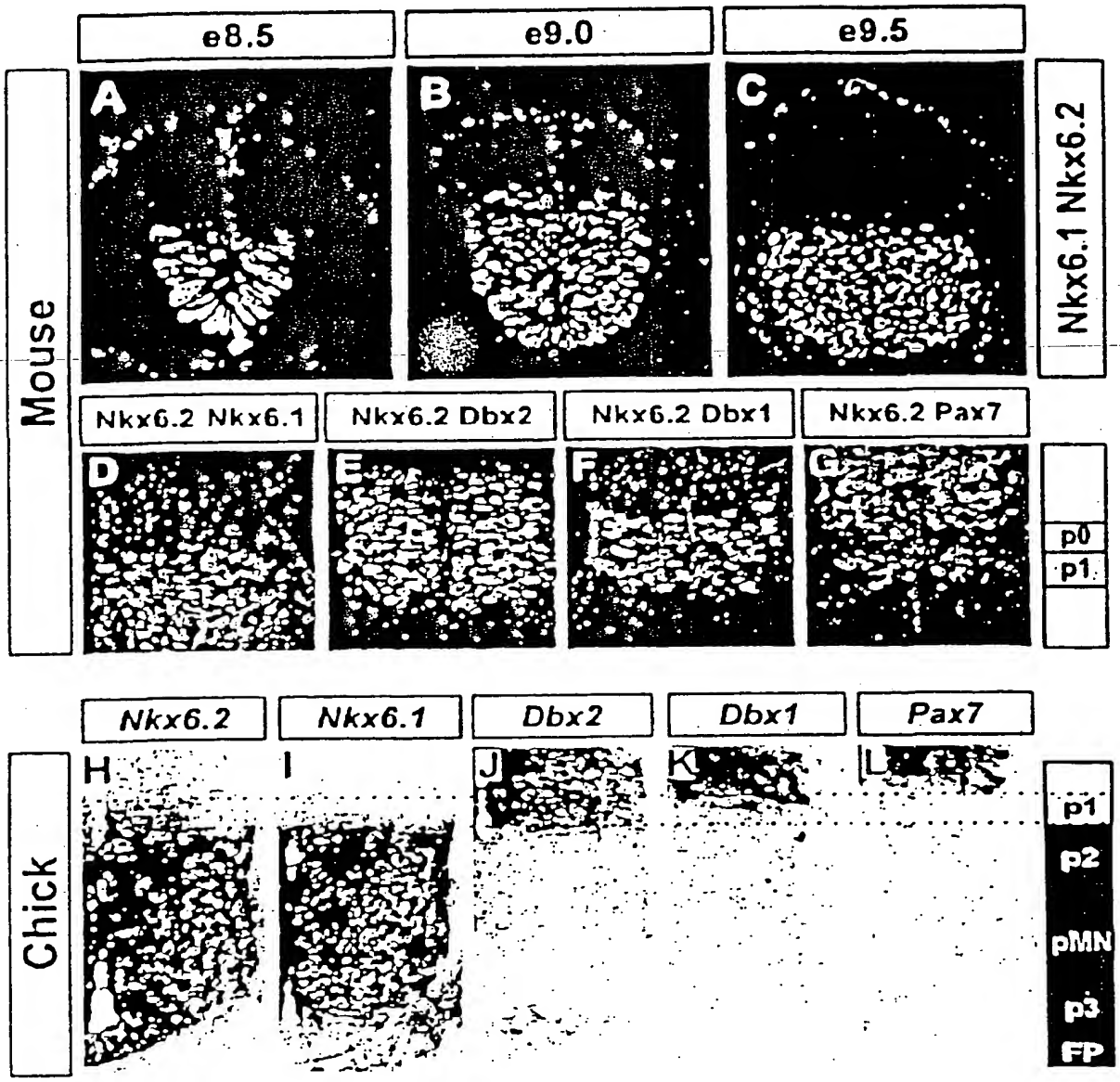
e8.5
e9.0
e9.5
Mouse
A
B
C
Nkx6.1 Nkx6.2
Nkx6.2 Nkx6.1
Nkx6.2 Dbx2
Nkx6.2 Dbx1
Nkx6.2 Pax7
D
E
F
G
p0
p1
Nkx6.2
Nkx6.1
Dbx2
Dbx1
Pax7
Chick
H
I
J
K
L
p1
p2
pMN
p3
FP

FIGURE 11

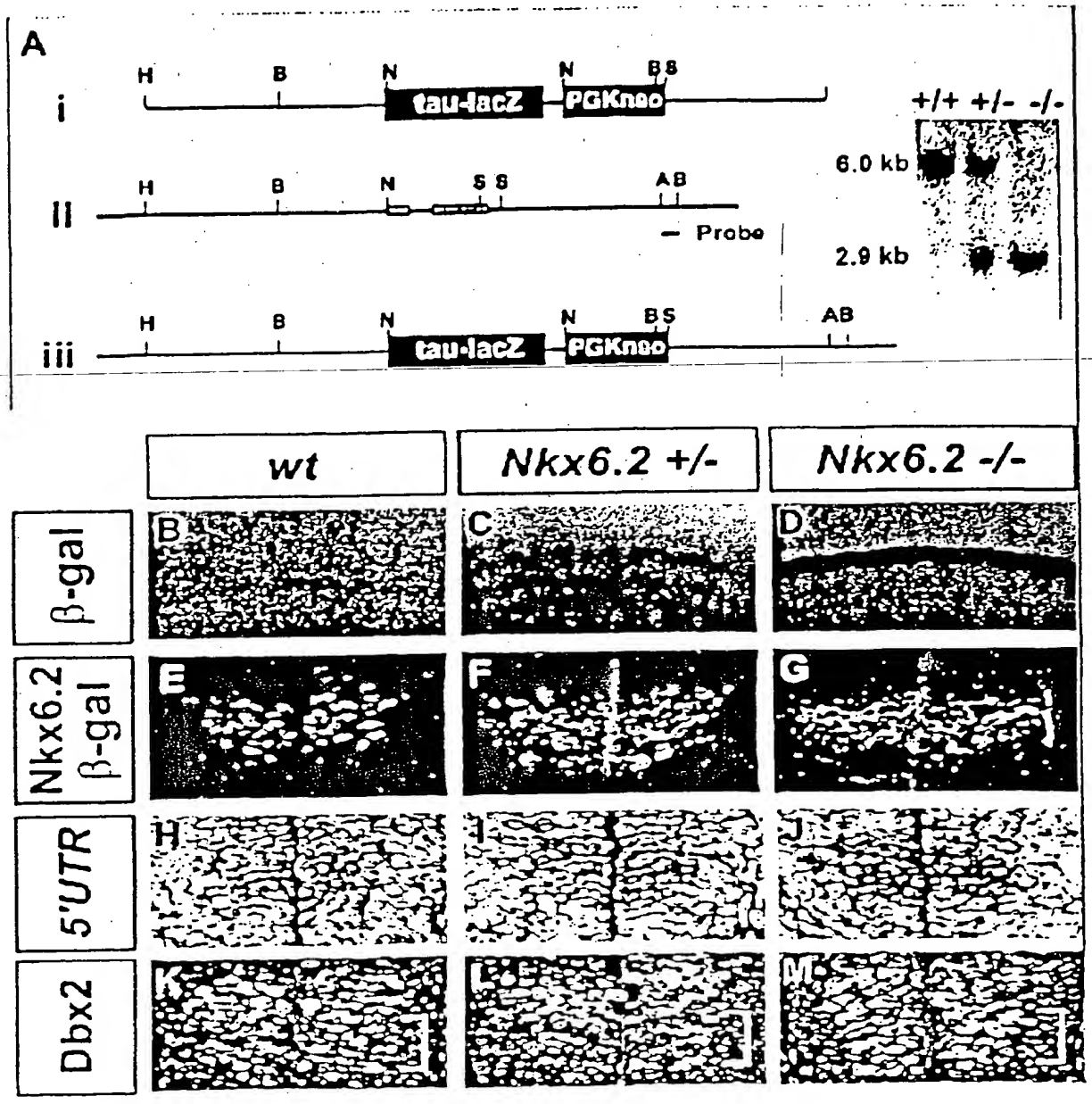
A
i
H
B
N
N
BS
tau-lacZ
PGKneo
+/+ +/- -/-
6.0 kb
2.9 kb
II
H
B
N
S S
AB
Probe
iii
H
B
N
N
BS
AB
tau-lacZ
PGKneo
wt
Nkx6.2 +/-
Nkx6.2 -/-
β-gal
Nkx6.2 β-gal
5'UTR
Dbx2
B
C
D
E
F
G
H
I
J
K
L
M

FIGURE 12

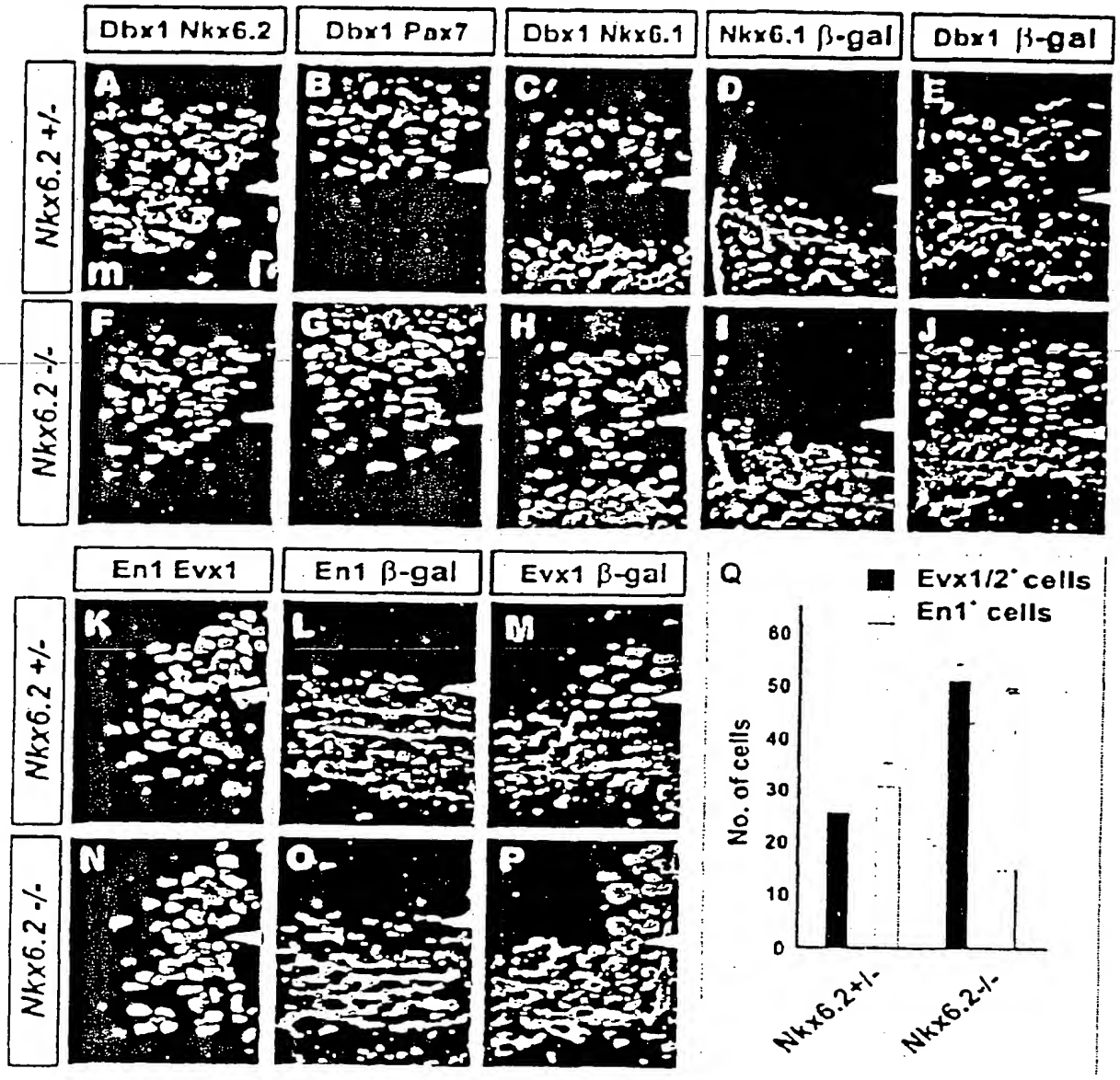
Dbx1 Nkx6.2
Dbx1 Pax7
Dbx1 Nkx6.1
Nkx6.1 β-gal
Dbx1 β-gal
Nkx6.2 +/-
Nkx6.2 -/-
A
B
C
D
E
F
G
H
I
J
En1 Evx1
En1 β-gal
Evx1 β-gal
K
L
M
N
O
P
Q
Evx1/2+ cells
En1+ cells
No. of cells
60
50
40
30
20
10
0
Nkx6.2+/-
Nkx6.2-/-

FIGURE 13

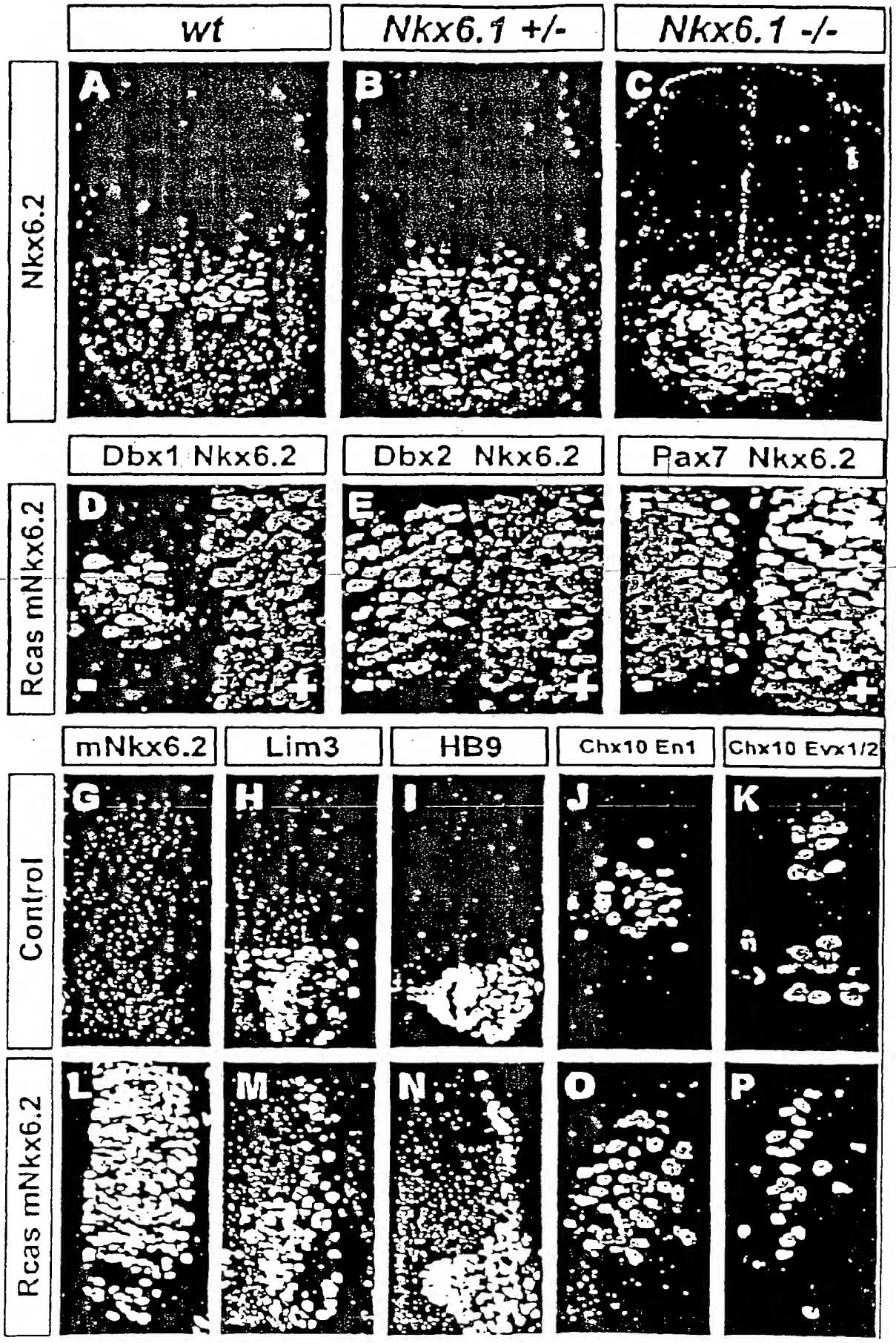
wt
Nkx6.1 +/-
Nkx6.1 -/-
Nkx6.2
A
B
C
Dbx1 Nkx6.2
Dbx2 Nkx6.2
Pax7 Nkx6.2
Rcas mNkx6.2
D
E
F
mNkx6.2
Lim3
HB9
Chx10 En1
Chx10 Evx1/2
Control
G
H
I
J
K
Rcas mNkx6.2
L
M
N
O
P

FIGURE 14

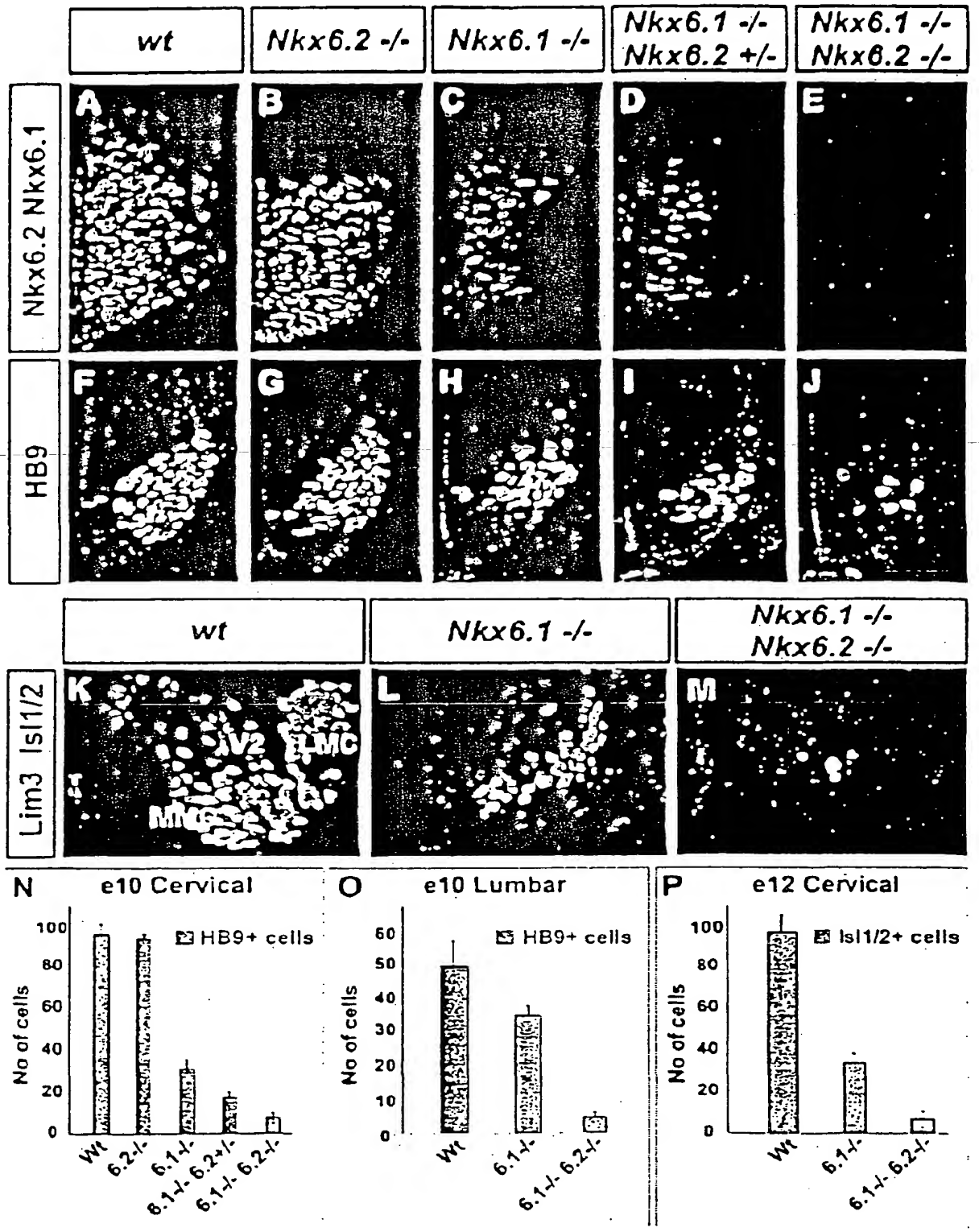
wt
Nkx6.2 -/-
Nkx6.1 -/-
Nkx6.1 -/- Nkx6.2 +/-
Nkx6.1 -/- Nkx6.2 -/-
Nkx6.2 Nkx6.1
HB9
A
B
C
D
E
F
G
H
I
J
wt
Nkx6.1 -/-
Nkx6.1 -/- Nkx6.2 -/-
Lim3 Isl1/2
K
L
M
V2
LMC
MMC
N
e10 Cervical
HB9+ cells
No of cells
100
80
60
40
20
0
Wt
6.2-/-
6.1-/-
6.1-/- 6.2+/-
6.1-/- 6.2-/-
O
e10 Lumbar
HB9+ cells
No of cells
60
50
40
30
20
10
0
Wt
6.1-/-
6.1-/- 6.2-/-
P
e12 Cervical
Isl1/2+ cells
No of cells
100
80
60
40
20
0
Wt
6.1-/-
6.1-/- 6.2-/-

FIGURE 15

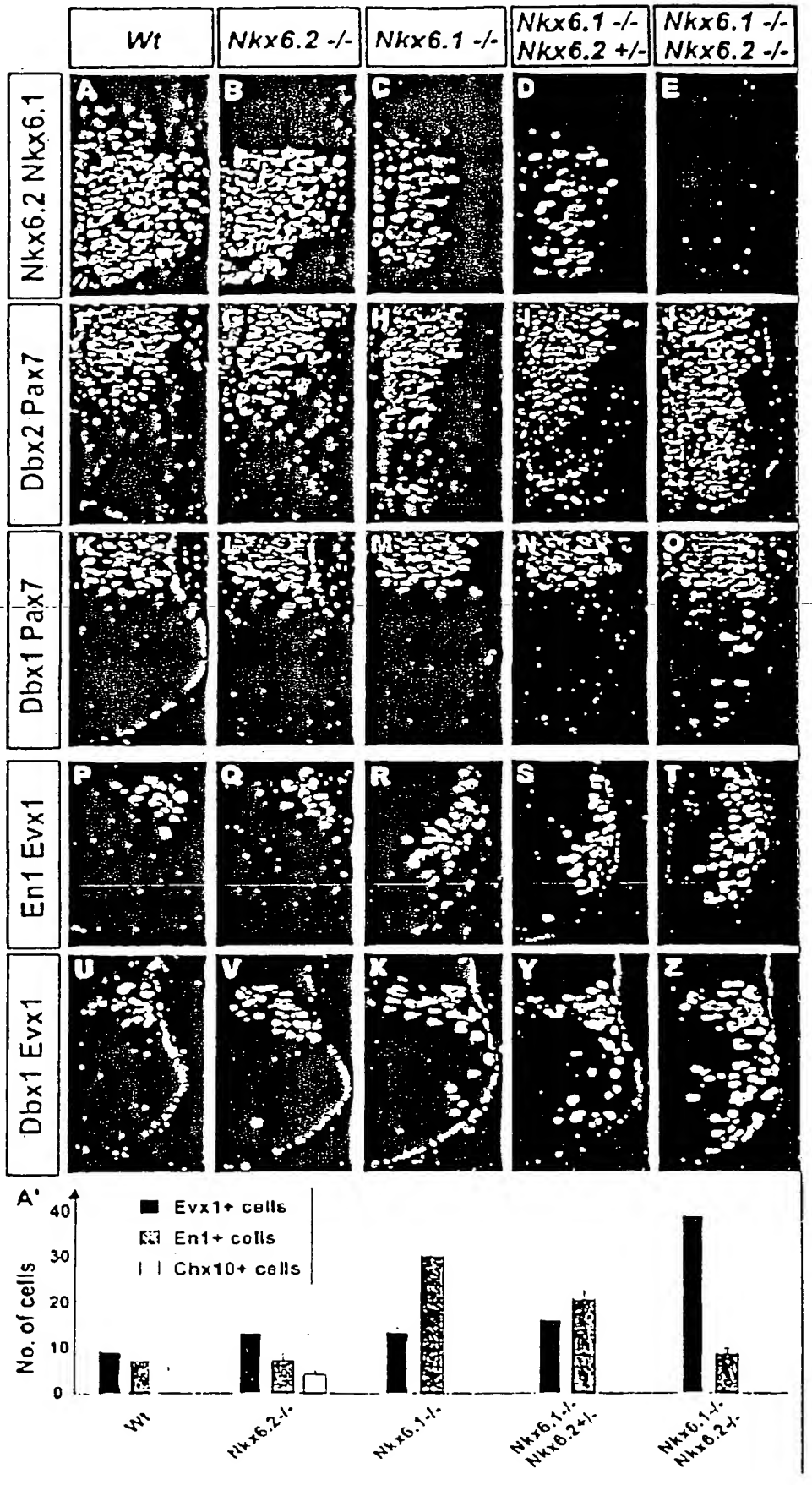
Wt
Nkx6.2 -/-
Nkx6.1 -/-
Nkx6.1 -/- Nkx6.2 +/-
Nkx6.1 -/- Nkx6.2 -/-
Nkx6.2 Nkx6.1
Dbx2 Pax7
Dbx1 Pax7
En1 Evx1
Dbx1 Evx1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
X
Y
Z
A'
Evx1+ cells
En1+ cells
Chx10+ cells
No. of cells
0
10
20
30
40
Wt
Nkx6.2-/-
Nkx6.1-/-
Nkx6.1-/- Nkx6.2+/-
Nkx6.1-/- Nkx6.2-/-

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FIGURE 16

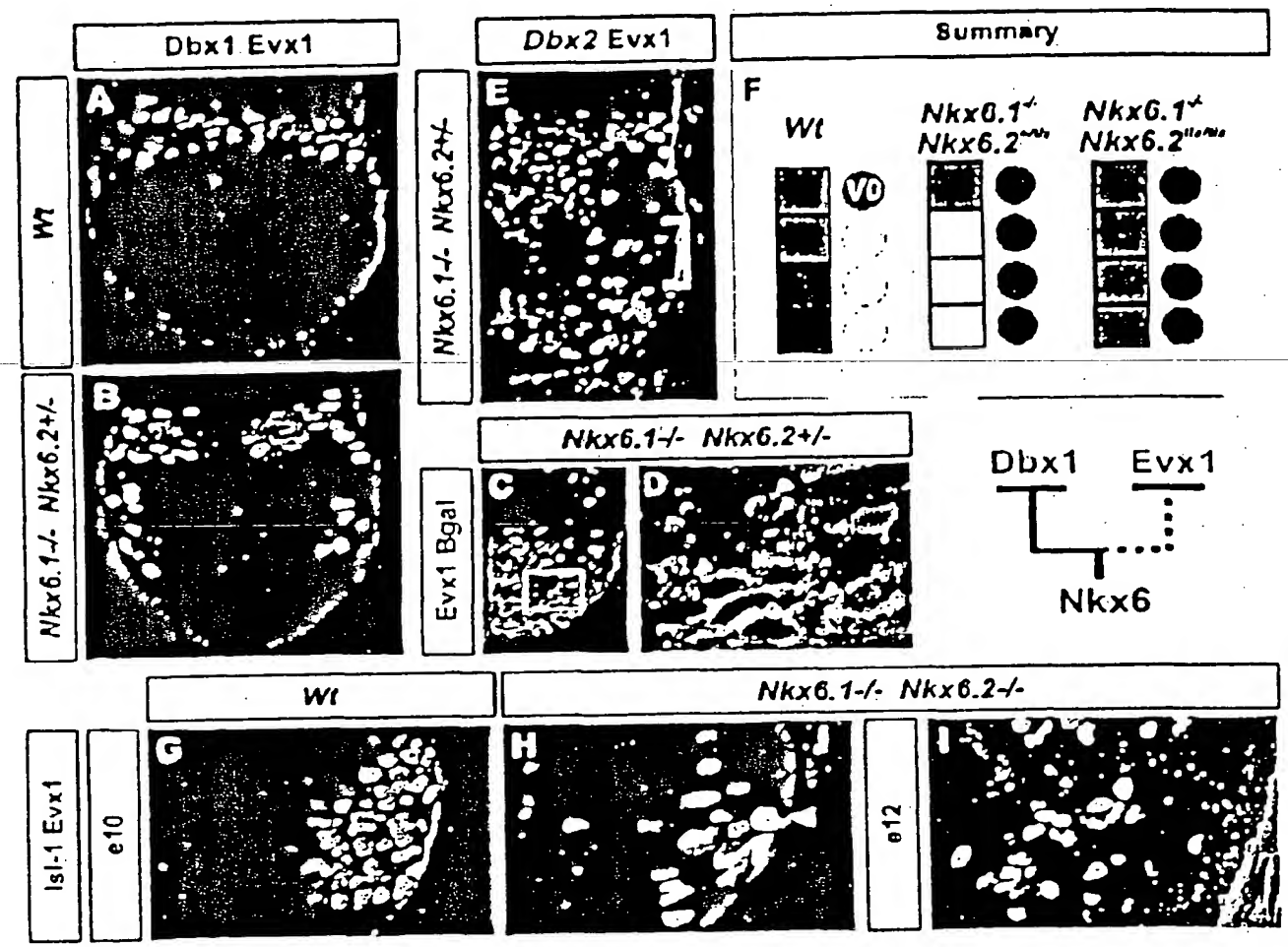

FIGURE 17

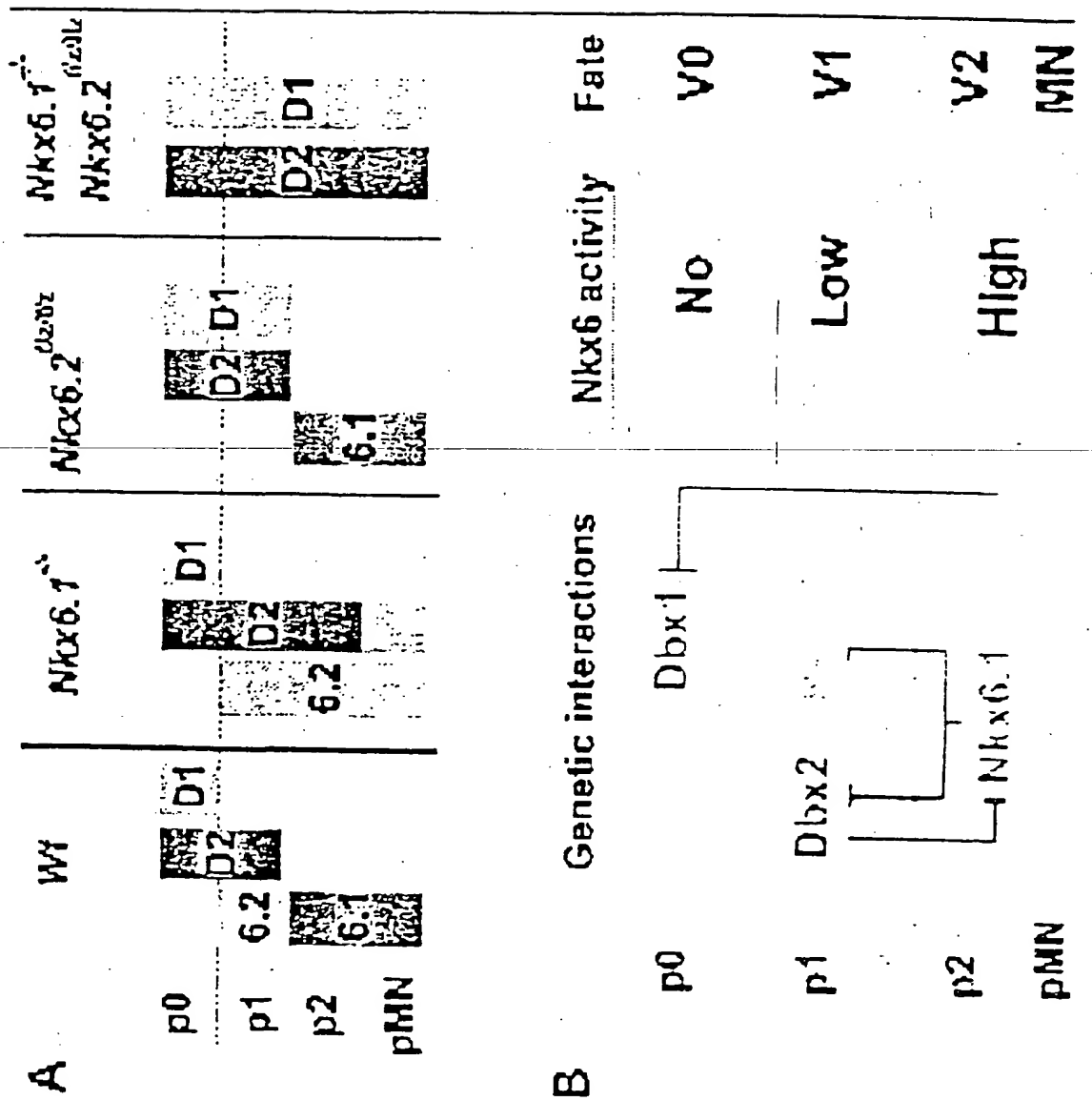
A
Wt
Nkx6.1-/-
Nkx6.2-/-
Nkx6.2-/-;Nkx6.1-/-
D1
D2
6.2
6.1
p0
p1
p2
pMN
B
Genetic interactions
Nkx6 activity
Fate
No
Low
High
V0
V1
V2
MN
Dbx1
Dbx2
Nkx6.1

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FIGURE 18

```
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  61 ctcttggcag caccgtgggg ccctcagagc cctccatcta gttccgggca gggcagggcc
 121 ccttcccaac accatcggct gcctctggtc actcccaccc aggggcacag ggaatctctg
 181 aacacccctt ccctggggag caggaagact tgaaacctcc ttggccaggc cagggcggtt
 241 tctactgtgc ccaccagacc cccaggctaa gccagcaggg agactggaag gcccagtgtc
 301 cagcccctgc cctgcctcag ggtggctgct cgccctctcc cctcccaccc cacctggaca
 361 gcctcggtcc tcagggcgct gtanggagtg aggcacctgt gggatggagc ctcagcgtgg
 421 gtgggagaga tgctgcaggg cccaggtgcg gagccgcgtg tgaacgggca ggcggcccgc
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 541 gaagaacttg gttgttcagg ggaagaactc ggaccattct ctgtgtgtct ctctctgtgc
 601 tttcggtaag tgctgctccs ttggctgctg catttacaag tgacttttaa agaacagaag
 661 ctggaaggaa acctggggct caggattcag ggaggggggcc ctgcaaggtg ggaggggccc
 721 ggccagggcc caggctgtgc aaggaacttc aggcccagtg aagctagagg gtccacaaag
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 961 ggcacccact gtggccccac cccagcttca tcaaagcctc cctccctgtc ctgggccaht
1021 cggcctgggg aaaagcggca ccctctcccc agagcctgat cctccamttc atgtggacct
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1501 atgtcctgag caaggtggcg aggaggcgga catcagacac atccactaat gccttcggca
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1921 ccacccctgg gcggtcccca ccccagccct cagcgctcat ggcctttcag acccggctgg
1981 gtccatgagc ccagtgggac gccggggctg cctggctggg atctgcgcct gcctcccagc
2041 cctttcccgc tgccctggca gggctgcccc cagagggcac gggagatggg gttggggtct
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2581 gcctgggcgg ccgaaggcga acgcaggctc cttcctctg tggagttccc ccgtcgcccc
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2761 ttgctgctct gacccgcccg cgcccgggcc ggagcccgct gcgttcacgg tgcacccccc
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2881 aatggcctct gccaaaaccc gcgctctcgg ttttccarcg cggagcgttc gcgccgatgc
2941 cggcagcctt cctcgcggga catctgctcg cgggccagga ggtggcatcg cggacctctc
3001 caggcagcgg ggccgccggg cggcggagcc caggcaaaga catcgcggtc kgaggcgctc
3061 ggaccttccc gggaggaggg ggagttgcct cggtggtttc cgagagggcg gcaccggggg
3121 acgcaggaga aaaggtgcgg gcgggggccg gagaggggac ggggcccgga gtgggcgccg
3181 ggaagcgtag gaggtgaagc caccggaccc acgcgcagct cggcgaggag gagggcggga
3241 aagcgcgtgg ggcgcaggcc ggggagccgg gtaggacgcg gcacctgcgg agcgcgccaa
3301 gacttccacg gcttacaaga acgtgggaga gggacccccc cttacccggc tcctctgcgc
```

FIGURE 18(CONT.)

```
3361 ccccaactca ccctggcccc tcatcccgcg cccctgagcc ctggagagcc gcgcgctccg
3421 cagccagtga cacgccagcc ccacccgcga ccccacgcgt tccctcggca gcccagggag
3481 gaccgggggg cgcagacaga cccagggttt atcgggccgc agcgcagcgc ctccaggtcc
3541 atgtttcctc agccataaaa cagcgctagc gacgcccccc gccccgcccc tccaggctgt
3601 gagaagagcc agagcctgtc ccgagcgcgg cttcctcccg ccgttccgcc cgcgcgcgcc
3661 tcctgggcct cagttcggga gacccgcacc cctcccggcc cgcccagggt ctgcccggcc
3721 gcgcagagtg ggggatccca gggcgacagc agcccccgcc cccaactccc cctgcccgcc
3781 ccccccggcc ccgcagttcc cgcgtctcag ctcagagccc gagccttggg cgcgggcgcc
3841 gtcgccttgg ggtgctggga ggggcccgaa cccgacccgg gagggccctt cctcgtgtct
3901 cctccgaggg agcggggcgc aggacaggcc ggggcgggtc tcggggccgg acgggcgctg
3961 ggggttcccg ggccaggctc cgcgggggcc gatcaccggt ggggcggccg cgcccaatcg
4021 aatcccaatc ccagtcgaat cgagtgcgga gtcgacgggg gaagcgaacc ccccgtgaac
4081 gcggggctgc acctcagtgg agccggaaag ccgccggggc agccccgagc gcgcacacac
4141 ccggcggccg caccactgcc ccggagtttg gccgcaggtg gcttttccaa gccgccatcc
4201 aggagcggcc gacggcgcca agtccccgcc tcgacctgca caaaacgaaa acggacgctg
4261 gaggggggcg agggggcgga cgtgagaccc cggccccgaa ccccgggcgc cgccttcctc
4321 cgcggcacag gcccgagaga ggccacgcag cggcgttccc tgcgcacaga ctcgggctcc
4381 ccacgagccg tgggccacag ccacagccgc cccgtgtccc taaatcaata cgagacgtca
4441 ccacagacgt cggagcgttt gctcgcggcc gccgtgcgcg gggctcggag tcatctcacc
4501 gcccggtctg cgggatggat gagcgagcgg ctcccggtgc cgtgggggcg gggggacac
4561 cggcccccg cgcgcgtcta aggccgcgtt tctgccgctg cgcccccagc ccgcacccac
4621 gttcgggccc tggacagggc ttccgcgctg aggccgtcct ggtctgtgtt ctcccggccg
4681 gggattcgcg agaggcggcc cgtgggcgaa gtcgtgggcc caggtcacat cctgggggac
4741 ccccagcggg agacctggag gccgatgacg gggaagtgcc gagccgcgcg tgtggtcccg
4801 ggacccgcct ccccgccccg ctcccgcctg cctcactcct ccaccgcgcc ggccgcgtgt
4861 cggcgaaacc agaggcagct ccgtgcgagc ctcgcccggc cgtgaggccc gtggattccg
4921 tggactcgag gcccgcgtcc tccgccctcc tgtggccccg acctgcccgg agcgcgttcc
4981 ccgccggcgt ccgctgccgc tcacacccac cccagccacg ggcggcggag cagtcgcgac
5041 tgggacgcgg gccgggactc ttccccgagt ggggcgctcc gagcgcgcgg gcgggtcctc
5101 aaatctgcat tctttccgtt aataaaatac gttctcgtat tttttcctga tttcgcatga
5161 aaacctttgc ctaactacac tcccatccaa gcgggattta tttcgtcccc ggggagataa
5221 atcggggcga atttacagcc cgggaggcac ctgccgcgct aatgggccct tcatggagtg
5281 cgcggccggc gggggcgcgc gggcgggggg ggggcgccgg ccaatggccg gaccgcgggg
5341 tccgcagcca atcagcgcgc gcgccgcgcc ccggcggagc ccccgttatc agcgcgtccg
5401 tcccgcgcgg cgccgctccg accggccccg ggagccgccg ccgccgccgc ccgcccgccc
5461 gccccgcgcc ggagccgccc gcccgccccc cgcgccccgc gccccgcgct gcagccgacg
5521 cccgcccggg ccgcgcgcaa acttcccggg ccggcgggca ggggcggcgg cggcgggccc
5581 cggatgggag cccgggccgg cggcggcggc gcccatggac actaaccgcc cgggcgcgtt
5641 cgtgctgagc agtgccccgc tggccgcgct gcacaacatg gccgagatga agacgtcgct
5701 gttcccctac gcgctgcagg gtccggccgg cttcaaggcg cccgcgctgg ggggcctggg
5761 cgcgcagctc ccgctcggga ccccgcacgg catcagcgac atcctgggcc ggcccgtggg
5821 cgcggcgggc gggggcctcc tggggggct gccccggctc aacgggctcg cgtcgtccgc
5881 cggcgtttac ttcgggcccg cggccgctgt ggcgcgcggc taccccaagc ccctggccga
5941 gctgccgggg cgcccgccca tcttctggcc cggcgtggtg cagggcgcgc cctggaggga
6001 cccgcgtctg gctggcccgg gtgagtggcc cgcgcggggg gtgcggggcg ggtgggcgcg
6061 gaggggggacc ccgccggccg ctgacctccc tcccttcccc tcccttgcag ccccggccgg
6121 cggcgtcctg gacaaggacg ggaagaagaa gcactcgcgc ccgaccttct cgggccagca
6181 gatcttcgcg ctggagaaaa ccttcgagca gaccaagtac ctggcgggcc cggagcgcgc
6241 gcgtctcgcc tactcgctgg gcatgaccga gagccaggtg aaggtgagcg cggcgggggct
6301 cgggagagca gagccgggggg cccgcgtcct gcgaacggcc ccagcgccag ccccgggccc
6361 cgcggccgcc tgaccgcccc gtccactccc aggtctggtt ccagaaccgc cggaccaagt
6421 ggcgcaagcg gcacgcggcg gagatggcgt cgccaagaa gaagcaggac tcggacgccg
6481 agaagctgaa ggtgggcggc tcggacgcgg aggacgacga cgaatacaac cggcccctgg
6541 accccaactc ggacgacgag aagatcacgc ggctgctcaa gaagcacaaa ccctcgaact
6601 tggcgctggt cagcccgtgc ggcggcggcg cgggggacgc cttgtgagga cccgcggggt
6661 gggggcgaat ctatttttgc agaatccggg ggcggccccg ggtgggcgcg agtcgctttg
6721 tatcatcaat aaattattta acgggtcccc gtcggagccg tcgctccgga gcctgcgccg
6781 cgtgtttctt ccgtctcgaa cccggagcga ggcggccccc ccccggcccc ggcttcgccc
6841 ctgcgcccgc ctcgggtcct ccgggttccc ggtgcggasg ctgcgggccc cgggcaggcg
6901 cgaggaggcg gcgaaggcgc agggaagggg cccggcccgc gggaaggaac cgcagcgaca
6961 gccgccagga gcccgggrcg gmgccgggga cggagcagca ggtacggccc ggcccgcctc
7021 gcctcggggc ggattcggac gcgcttgggg gttcccgaaa gggcgggtga gccgcgtacc
7081 cgcctcgagt ccccgcggga ggtttttctt cttccgtttt cccgctttgg ggccacgtac
7141 tcgttgccac cgggcacccg ttcccgctcg gccgagggct tcgctctgat tatttccaaa
7201 gtccctctgc gcatcagcgg atcccatagg cccgccctgg gctcagccgg tggaaccggg
7261 tctgatccgc tgcacggagg cccttcggtc accatcccgc cagatcttcc cgcggtggaa
7321 agcagtttct tccgaactag gaccgcaaag agaaatccga aataattccg cccgcggagc
7381 ggcggggcct cccgtgggtc acgcggggtc agggagccgg aggccccctg ggcaaggccc
7441 gcaagcgccc agccgggggg ctcgggggac ccgtcttcct gccctgaaat gccgccagct
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FIGURE 18(CONT.)

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 7501 ccgccggggc tgtgactgcg gctgacaaaa cccctccarc ctcccgcarc ctctgttggc
 7561 cggggctgcc matccgctgc awcttaatgg gcgtggctgt tgagttttaa tttttaaaaa
 7621 ttaaatgtaa ataatgatat cactgcggtg gtacgatttc tcttggcatt tgcggaagcg
 7681 ttaaagggaa atagaaaggg cttaaactcg gcgcgttttg ttttaggctc ttagcagcct
 7741 tctttacaag gaagcaactc gaagggcaga agcaacgctt ttctgtgggg agcccctctc
 7801 agctcagagc agaggggctt cttaaagttt tgaggaaggc aaagcgttga tataatcccg
 7861 ttttaaaatg ttgagggata aatcctttat tacagtagaa agtccaaaag gctgtgtttc
 7921 tcctctcaat gaacggctta gtgttttgtg acagcgtgtg atacagtgaa attccaggat
 7981 ttctaatgag cttgatctca aataaaggct atacangagg ccgctcccct gagttagcat
 8041 ttcaaaggtg gcaggagaag ggaaaggaag aaaaagcaac acggggacta ttttcaccac
 8101 ggtcaatttt attgcttagg aaccagaccg gtcacttcca aaggcccctc agaacgacca
 8161 acagctgaaa cccgcggggc ggactccgtg ttgaaccgcg gacagcggca accacagcag
 8221 cgacacggac ctgtgcttcc accaagaaca gattccgcag cggacagcag tcacttgcag
 8281 tggtagtatt tatcccacac aaacacccag ctaatgcctt caccccggtc ccaggaactc
 8341 tgtagtgttc taaagtaaaa atcaataaaa acatacattt gtgtttcatc aacagactct
 8401 ctaatcacct tctaatgctg tacttactgc tataggagaa aaatatttgc aacaaggtta
 8461 tgacatgggt tgtctgtagc ggagcaatga ggaaatgtac agttttgttt ctctttaata
 8521 ttttatatta cagcccatgt taaaagcagt ttctattgga agcaaactag gctatttcta
 8581 tttctcccat gatattattg ttgtaacgta ggatacttgg caccataaaa cagtaacaaa
 8641 agacagacaa acggtttaca aaattcttaa aaggtacacc caggctagct ataaacttca
 8701 cattcagttc ttaatattac acagaagaac ggcatgggag taacggcccg ctggtgcaga
 8761 cgtgctgtgg ggccgatttt acccacgatg gcgaggccat gtgtgttttt tacgaatttg
 8821 tgtgttgatg gacacacagc tgagctccta gactccaatg ccgcctgctg atgggactct
 8881 cctgtgcgtt catactggaa agtatattta gcataagttt tggtaagatt tataaattat
 8941 ttttaaaaag tatatattta tatatattta tatatatata aaaatggaaa gcagctgcag
 9001 tgtgattcaa aaaccatgtg acacggcgca gagtcagtgc cgcggaagga gcatcggcag
 9061 agacagaccc ccttgccatg ctcagggcca cgctgccggc cggcagaggg agtgcccgtc
 9121 tcggcttccc cagcccctgg acacacctcc acctggcaga gggggtccct ggacacagtg
 9181 gggggtctct gtgctgaaga agcccctcca ctggcaatca ttaaaaactg aaaactgtga
 9241 agtctacggt acagaccctc tttgctgtct attagagttt tgacaacagg actgtgactt
 9301 atttaaaaaa aaaaaaaaac caatatttct acttaatgtc acatagacag acgagacagt
 9361 gaggtatgtg gggctgctcc ggaatggtcc ggaggctgaa gcgaagtgtg gggctggccg
 9421 tctagcaggt ggcgcttggg cgggttctcg atgcagcttt caagagtgcg tattcggtcc
 9481 acggctacag ggaggctcac gaagtgtcct ctcgtggcgc tggcatctct tcccaccacg
 9541 tcactgcacg acacaacact tgtgcacatg ggcatgaggt ttacctgccc cgggcatgat
 9601 tcggaaggcc aggaacacgg gcttgtggtc tcccatgcag acgttgggcc caatgtggtc
 9661 ataggtgaca accttctcct cgctctccga ccgcagcacc agctccttgg cagacgggga
 9721 catgaggatg cggtcacacc aggctgggca ccgggtgttc atgtactgct caccctggcg
 9781 ggcgtcctca ctgtacgggt agctgggagg gaacgagatg tccagttcat acagtctgtc
 9841 cttaaagaca gacaactcct tgtcaaactc caagagcgcg gtgccgttgt tgtctcggaa
 9901 aacctcctgg ttgaagtagt cgaagagttt cttttctaac tggagcataa ccttccggtc
 9961 gttgtccgac tcacgaaata tgagcttcac cacttcattg gtgtcggcgg cccggaccgt
10021 ctgcatcggt ggttttgctg agagcgtctc cacgacagac ttggaatcca gccggaagtt
10081 gaaatcacca aatacaaagt aggaaacctt ctcgaatcgc tgatcaatga ttctgtccag
10141 cacgtagccc agtgccttgt gccggattcc cgagtacacg gaagggcttg tttcccaggc
10201 gaccagattg gaagcatcat ggaaaagatg gatattcacc aagtcaaagg cacagtctgc
10261 aatcaccacc tcgtccggat gaagcctttt cttgaccatt tgcactcggg gaagtagtct
10321 gcgaaacttc tccttctcca gcatgggcgt gctctctaag gtatccgagt agatctcttt
10381 gccagcgacc tttctatact tcttagcttt aaagtcaaac tggtagatgt tttttaagga
10441 ctcatgaaga aaataaaagc tt
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FIGURE 19

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  1 mdtnrpgafv lssaplaalh nmaemktslf pyalqgpagf kapalgglga qlplgtphgi
 61 sdilgrpvga agggllgglp rlnglassag vyfgpaaava rgypkplael pgrppifwpg
121 vvggapwrdp rlagpapagg vldkdgkkkh srptfsgqqi falektfeqt kylagperar
181 layslgmtes qvkvwfqnrr tkwrkrhaae masakkkqds daeklkvggs daedddeynr
241 pldpnsddek itrllkkhkp snlalvspcg ggagdal
```
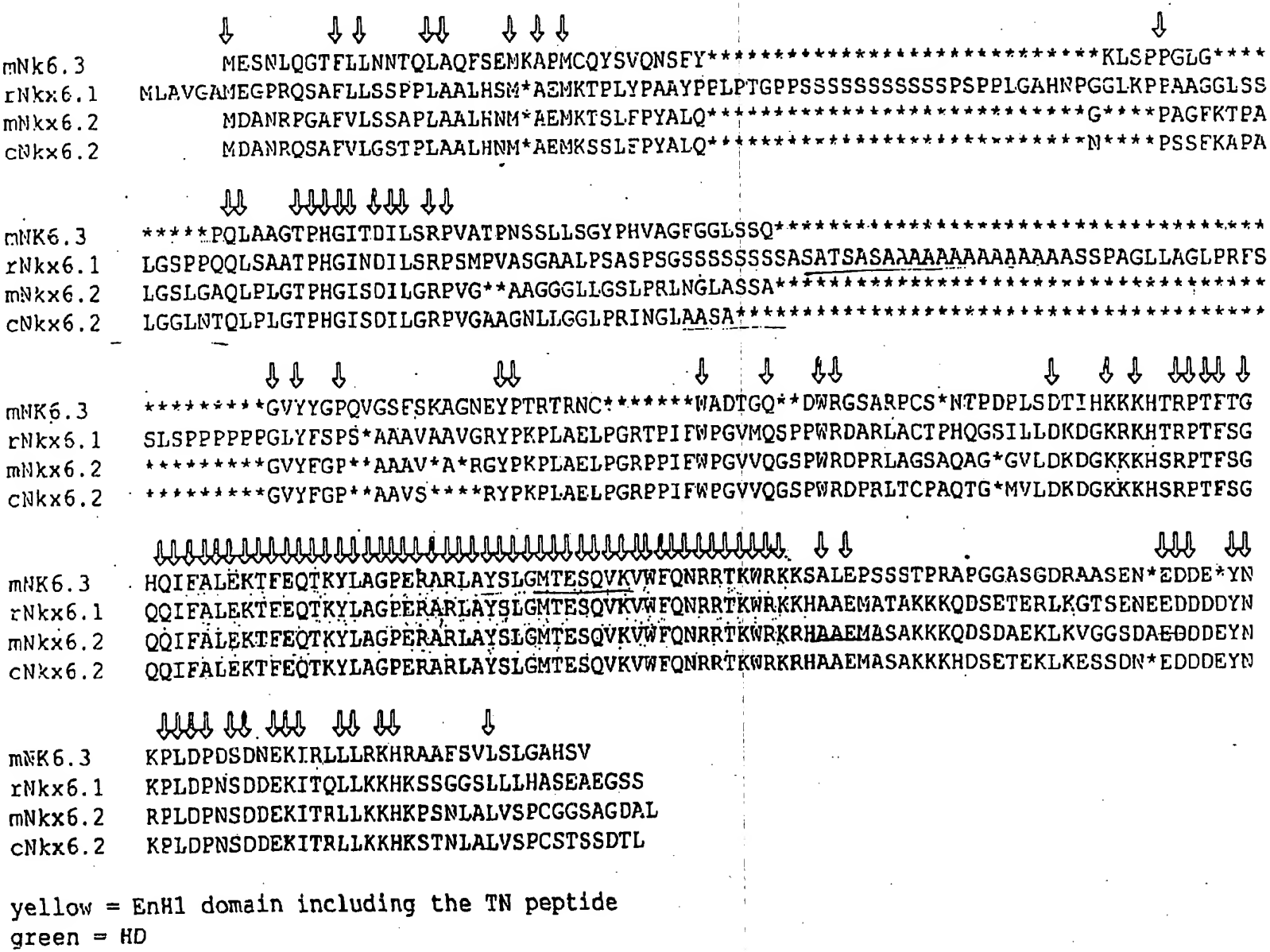

FIGURE 20



yellow = EnH1 domain including the TN peptide
green = HD

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/27256

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 5/00, 5/02, 15/63, 15/85, 15/87

US CL :435/4, 325, 366, 368, 455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 325, 366, 368, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRISCOE et al. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell. 12 May 2000, Vol. 101, pages 435-445, see entire document.	1-24
A	MIRMIRA et al. Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. J. Biol. Chem. 12 May 2000, Vol. 275, No. 19, pages 14743-14751, see entire document.	1-24
A	OSTER et al. Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. J. Histochem and Cytochem. 1998, Vol. 46, No. 6, pages 717-721, see entire document.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 NOVEMBER 2001	15 JAN 2002

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Janice Ford for

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/27256

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	SCHWITZGEBEL et al. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. 2000, Vol. 127, pages 3533-3542, see entire document.	1-24

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INTERNATIONAL SEARCH REPORT

Int[illegible]nal application No.
PCT/US01/27256

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST
Dialog (file: medicine)
search terms: Nkx6.1, Nkx6.2, briscoe, ericson, rubenstein, sander, stem(w)cell, neuron?